

THE AETIOLOGY OF THE PROLIFERATIVE ENTEROPATHIES

STEVEN McORIST

Ph.D. THESIS

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DECLARATION

I declare that the work incorporated in this thesis has been performed by myself, except as stated in the Acknowledgements, and that the thesis was composed by myself.

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ABSTRACT OF THESIS (Regulation 7.9)

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The enteropathogenicity and antigens of Campylobacter-like organisms and Campylobacter spp associated with the proliferative enteropathies were investigated.

Two gnotobiotic pigs exposed orally to a filtered suspension of intestinal mucosa designated 284/86 from a naturally infected pig subsequently developed lesions of proliferative enteritis. Culture of the successful mucosal inoculum only revealed a moderate number of C. coli, however an apparently greater number of Campylobacter-like organisms was evident in smears of this inoculum. The pathogenesis of porcine proliferative enteritis was clearer from the results of this study. Ten days after infection, curved bacilli had colonised the ileal and large intestinal crypts. Attachment and entry of Campylobacter-like organisms into crypt enterocytes was also evident, with some proliferation of both bacteria within cells and of the enterocytes themselves. Twenty days after infection there was similar intracellular colonisation of bacteria and proliferative activity, although no luminal bacteria were evident. A moderate sub-acute inflammatory reaction was evident throughout.

Conventional hamsters dosed with C. jejuni developed varying degrees of localised acute intestinal inflammation. Hamsters dosed with C. hyointestinalis or C. coli did not develop any lesions. Lesions of proliferative enteritis were detected in hamsters dosed with porcine tissue 284/86. Numerous intra-cytoplasmic Campylobacter-like organisms were detected within enterocytes in affected portions of intestine. Weanling hamsters thus proved to be susceptible to the agent of porcine proliferative enteritis by cross-species transmission.

Whole cell antigen preparations were made of various Campylobacter sp. Indirect immunofluorescence assays incorporating rabbit antisera to each Campylobacter sp gave specific endpoints for each antiserum of 1:160 to 1:320. Rabbit antisera prepared to Campylobacter-like organisms partly purified from proliferative enteritis mucosa, by a homogenisation and filtration technique, also gave specific reactions in this assay, up to 1:640. Intracellular Campylobacter-like organisms were also compared in gel electrophoresis protein profiles and immunoblotting reactions to Campylobacter spp. The intracellular organisms tested had a distinctive protein profile dissimilar to the profiles of the known Campylobacter spp. In immunoblotting reactions, each of the Campylobacter sp antisera reacted strongly with homologous antigens, but none reacted with Campylobacter-like organisms prepared from lesions, except for a minor reaction seen with one serum. Similarly antisera to Campylobacter-like organisms showed a strong reaction to 25K to 27K components of homologous antigens, with only minor reactions to various other components of the cultivated Campylobacter spp. Therefore it is likely that the intracellular Campylobacter-like organisms have a distinctive antigenic profile and that the 25 and 27K components are major antigenic components.

Mouse monoclonal antibodies were produced that were apparently specific to the intracellular Campylobacter-like organisms. Immunoblotting results showed that these antibodies only bound to a 25K to 27K outer membrane component present in the intracellular organisms. Reactions with this component could not be detected in assays with normal pig intestine, or Campylobacter sp antigen.

Restriction endonuclease digestion of Campylobacter sp with Bgl II gave suitably resolved DNA fragments of between 2kb and 25kb. Patterns obtained with Bgl II digestion of Campylobacter sp were dissimilar to those of Campylobacter-like organisms, and each Campylobacter sp had a characteristic distinct pattern. Digestion of DNA from porcine tissue samples with Bgl II produced a diffuse smear of fragmented DNA bands between 0.5 and 19kb, with no recognisable "ladder" effect. The genome of the Campylobacter-like organisms within enterocytes in proliferative enteritis therefore is different to that of known Campylobacter spp associated with the disease. This suggests that the differences in antigenic structures between these bacteria are due to genetic differences. Only a limited number of strains were examined.

Looking at the evidence provided by this study, the overall tenor of the results suggests that the intracellular organisms could be a separate, new species of Campylobacter. If indeed the intracellular organisms are a single, new Campylobacter species, then a new name may be proposed, such as "C. intracellulare". Verification of the validity of such a proposal would require further DNA studies.

CHAPTER 1

LITERATURE REVIEW

Introduction

Proliferative enteropathy or enteritis is a pathologically defined disease affecting a variety of mammals, frequently being clinically important in pigs and hamsters (Rowland and Lawson, 1986; Jacoby and Johnson, 1981). Consistent features are hyperplasia of the crypt cells of the intestinal epithelium (particularly those in the ileum), and the occurrence of intracytoplasmic, non-membrane bound Campylobacter-like organisms within enterocytes in affected portions of intestine (Rowland and Lawson, 1974; Johnson and Jacoby, 1978). Proliferative enteritis has been described under various synonyms, including intestinal adenomatosis and transmissible ileal hyperlasia.

Although it is often self-curing, proliferative enteritis in pigs is of significant clinical importance (Rowland and Lawson, 1986; Clark, 1987), the signs being anorexia, wasting and occasionally diarrhoea in 6 to 20 week old pigs. Subclinical disease can cause a significant reduction in the weight gain of affected pigs (Roberts et al, 1979). Significant complications of the disease include necrotic enteritis and a haemorrhagic enteropathy (Rowland and Lawson, 1975). Proliferative enteritis in hamsters is of major laboratory animal health significance in North America (Battles, 1985).

The identity and origin of the Campylobacter-like organisms within enterocytes during proliferative enteritis, and their exact relationship to its pathogenesis have not been resolved. Their consistent presence during the disease naturally suggests that they are the aetiological agent for the hyperplasia and inflammation (Wagner et al, 1973; Rowland and Lawson, 1974). However attempts at experimental reproduction of proliferative enteritis using pure cultures of Campylobacter sp bacteria as inocula have consistently failed. However if either proliferative mucosa, or such mucosa purified such that the

Campylobacter-like organisms present, is a major component of the inoculum, then proliferative enteritis can be reproduced in hamsters (Jacoby et al, 1975) and pigs (Roberts et al, 1977).

The general aim of this study is to elucidate the aetiology of proliferative enteritis in pigs and hamsters. This will involve investigation of the enteropathogenicity and antigens of Campylobacter-like organisms and Campylobacter spp associated with the disease.

Culture of Campylobacter sp from Lesions of Proliferative Enteritis

Culture of Campylobacter spp requires 5 per cent oxygen in the atmosphere of incubation, in addition some strains require 5 to 10 per cent carbon dioxide or 20 to 60 per cent hydrogen in this atmosphere (Kiggins and Plastringe, 1956; Lawson et al, 1981).

Campylobacter spp are non-fermentative, and derive energy by deamination of proteins into compounds easily incorporated into their Krebs's cycle, thus a protein-rich basal medium enriched with whole blood is optimal (Bolton and Coates, 1983).

The methodology for culture of Campylobacter spp from lesions of proliferative enteritis was reviewed by Lawson and Rowland (1984). Briefly, the ileal mucosa is homogenized to release intracellular bacteria, diluted to reduce contaminating bacteria, and samples of the dilutions are plated onto optimal media, with or without selective antibiotics. Inoculated media are incubated at 37°C for several days in an appropriate atmosphere. Various combinations of rifampicin, polymyxin and other selective antibacterial agents have been incorporated into such media (Skirrow, 1977; McCartney et al, 1984).

The filtration of crude faecal or intestinal samples through

filters of 0.65µm pore size to reduce contaminants prior to plating can remove the need for highly selective media (Boosinger et al, 1985); however this technique can suffer from technical problems (Lawson and Rowland, 1984).

Distribution of Campylobacter spp Among Pigs and Hamsters

C.jejuni is part of the intestinal flora of 0 to 100 per cent of healthy hamsters. The wide range is due to some groups of hamsters being free of infection, whereas others have a high rate of infection due to oral-faecal transmission, following the introduction of an infected animal (Fox et al, 1981; Fox, 1982). Similarly, C.coli is part of the intestinal flora of 70 to 100 per cent of healthy pigs (Prescott and Munroe, 1982; Rosef et al, 1983). C.jejuni/coli have been cultured from some lesions of proliferative enteritis in pigs (particularly haemorrhagic enteropathy) and hamsters (Rowland and Lawson, 1975; Lawson et al, 1979; Lentsch et al, 1982; Regina and Lonigro, 1982; Gebhart et al, 1983), but their common occurrence in healthy animals makes such isolations difficult to evaluate. The occurrence of both non-pathogenic and enterotoxic strains of C.jejuni is well recognised (Klipstein et al, 1985). Recently, monoclonal antibodies to C.jejuni/coli identified these organisms within the lumen of the intestine of hamsters affected by proliferative enteritis, but not within enterocytes (Stills et al, 1987).

The catalase-negative C.mucosalis (Roop et al, 1985) previously known as C.sputorum subsp mucosalis (Lawson et al, 1981) can be cultured from 52 to 94 per cent of ileal lesions of proliferative enteritis (intestinal adenomatosis) in pigs (McCartney, 1982; Lomax and Glock, 1982; Gebhart et al, 1983). The mean concentration of C.mucosalis in these lesions is usually 10⁶ per gram of wet tissue,

indicating a large number of bacteria per cell (Lawson et al, 1979; Ohya et al, 1985). C.mucosalis has not been cultured from the intestines of healthy pigs (Lawson and Rowland, 1974; Ohya et al, 1985). It can only be cultured from up to 10 per cent of intestines affected by haemorrhagic enteropathy (Lawson et al, 1979; Gebhart et al, 1983). However it has been cultured from the oral cavity of some healthy piglets, and the intestine of some diarrhoeic piglets (Gebhart et al, 1983; Lawson and Rowland, 1984). C.mucosalis appears to form part of the flora of the oral cavity of pigs. That it may thereafter affect the ileum to cause proliferative enteritis at weaning, was suggested by Roberts et al (1980a,b); experimental study undertaken by these authors did not allow any definite conclusions.

The catalase-positive C.hyointestinalis (Gebhart et al, 1985a) can be cultured or identified by immunofluorescence in 67 to 93 per cent of ileal lesions of proliferative enteritis in pigs (Gebhart et al, 1983; Chang et al, 1984a). It was also isolated from one hamster with "enteritis" (Gebhart et al, 1983). C.hyointestinalis has not been cultured from the intestine of healthy pigs (Ohya et al, 1985). However it is a common isolate from pigs with enteric diseases other than proliferative enteritis (Lambert et al, 1984) and from young cattle (Snodgrass et al, 1986). C.hyointestinalis and C.fetus show 30 per cent homology in the DNA sequences of their genomes (Gebhart et al, 1985a), indicating that they are more closely related to each other than to other Campylobacter sp. C.fetus has been isolated infrequently from pigs and has not been associated with naturally occurring enteric disease in livestock (Smibert, 1978).

The relative importance of C.mucosalis and C.hyointestinalis in the aetiology of proliferative enteritis is not clear from these data. C.mucosalis and C.hyointestinalis have been regularly isolated together

from proliferative lesions (Ohya et al, 1985; Wilson et al, 1986). In recent immunofluorescence studies either both (Chang et al, 1984a) or neither organisms (Lawson et al, 1985; Gebhart, 1987) were identified as being within enterocytes in lesions of proliferative enteritis in pigs.

Experimental Reproduction of Proliferative Enteritis in the Pig and Hamster

Transmission with crude mucosal homogenate

Proliferative enteritis was apparently reproduced in 4 of 9 neonatal piglets dosed once with affected mucosa from a naturally affected pig (Roberts et al, 1977). C.mucosalis was recovered in high numbers from the inoculum, the intestine of an exposed piglet prior to lesions being noted (day 38 after infection), and from exposed piglets with lesions of proliferative enteritis, day 51 to 59 after infection. Similarly, Lomax et al (1982b) reproduced proliferative enteritis in 15 of 22 ten week old piglets dosed four times with affected mucosa. C.mucosalis was cultured from the intestine of 7 of these 15 pigs, 4 to 5 weeks after infection. However the exact pathogenesis of proliferative enteritis in pigs remains unresolved as many other similar experiments have failed to produce lesions (Rowland and Lawson, 1986). Subsequent to the commencement of this study, Mapother et al (1987a,b) reproduced proliferative enteritis in a majority of four week old piglets dosed once with affected mucosa. Concurrent corticosteroid treatment had been given. C.hyointestinalis and C.coli were isolated from the affected piglets.

Several experiments have better defined the pathogenesis of proliferative enteritis in the hamster, but the aetiological agent remains unidentified. A majority of weanling hamsters dosed with cell-free fluid from crude homogenate of affected mucosa from hamsters

with proliferative enteritis developed lesions after 3 weeks (Jacoby et al, 1975; Frisk and Wagner, 1977). Both 0.22 and 0.45µm filters blocked this infectivity (Jacoby et al, 1975). Hamsters dosed with both infective mucosa and tetracycline had a greatly reduced likelihood of acquiring lesions (Regina et al, 1980). Hamsters dosed with either homogenates of normal hamster ileum or proliferative enteritis mucosa treated with heat (56°C) or chloroform, did not develop lesions (Jacoby et al, 1975). C.jejuni was the only Campylobacter sp isolated from infective inocula in one study (Regina and Lonigro, 1982).

Experiments aimed at elucidating the pathogenesis of proliferative enteritis found that non-membrane bound Campylobacter-like bacteria appeared within the cytoplasm of enterocytes in the crypts of the ileum 5 days after inoculation. Hyperplasia of these cells was clearly evident 10 days after inoculation, but only in those areas of epithelium parasitised by bacteria. Mononuclear cell infiltration of the lamina propria in affected areas of mucosa was evident 15 days after inoculation (Jacoby, 1978; Johnson and Jacoby, 1978).

In a recent preliminary study, 19 recently weaned hamsters were inoculated with affected mucosa from a pig with proliferative enteritis (Gebhart et al, 1985b). Thirteen of these hamsters developed proliferative enteritis after 3 weeks, with the controls remaining normal.

Transmission with pure cultures

Experiments designed to fulfil Koch's postulates for Campylobacter spp isolated from lesions of proliferative enteritis were reviewed by Roberts et al (1980a,b), Lomax et al (1982a,b), McCartney et al (1984), Chang et al (1984b), Boosinger et al (1985) and Gebhart (1987); who all then conducted such experiments, using pure cultures of C.mucosalis

and/or C.hyointestinalis, in conventional and/or gnotobiotic piglets. None of these experiments reproduced proliferative enteritis. While one study (Lomax et al, 1982b) reported that one of two piglets inoculated with C.mucosalis alone subsequently developed proliferative enteritis, the lack of experimental detail has made the validity of this result equivocal.

Whilst C.hyointestinalis persistently infects the intestines of gnotobiotic piglets following oral inoculation (Boosinger et al, 1985), C.mucosalis colonisation can vary in duration from 3 days to several weeks (McCartney et al, 1984; Boosinger et al, 1985). Only occasional intracellular Campylobacter-like organisms, and no enterocyte proliferation were detected in experimental piglets in these studies. Oral inoculation of conventional or gnotobiotic piglets with C.coli leads to persistent intestinal infection, with no significant lesions (Davis, 1961; Kashiwazaki et al, 1971; Olubunmi and Taylor, 1982).

One experiment designed to fulfil Koch's postulates for C.jejuni isolated from lesions in hamsters led to colonisation and persistent infection of the intestines of dosed hamsters, however lesions were confined to mucosal congestion and mononuclear cell infiltration (Regina and Lonigro, 1982). Similar infections have been noted in hamsters dosed with C.jejuni strains from healthy hamsters (Lentsch et al, 1982), humans (Humphrey et al, 1985), or dogs (Fox et al, 1986).

Reasons for failure of transmission experiments

Numerous factors could account for the lack of success of experiments including use of the wrong organism, animals of inappropriate age, breed, immunity status or diet. Neonatal pigs selectively absorb large antigens through their jejunal epithelial cells, and the lateral intercellular spaces between them, whereas

antigen uptake in weaned animals occurs through specialized and immature epithelial cells, particularly in the ileum (Walker et al, 1972; Owen, 1977; Staley and Bush, 1985). Also, the immune response to foreign antigens presented to the intestine is enhanced in the neonatal and post-weaning periods (Hanson, 1981). This "adjuvant-like" effect of weaning may be due to associated changes in the intestinal microflora and dietary protein antigens (Hanson, 1981; Stokes et al, 1983). However, the technical difficulties in raising gnotobiotic piglets to weaning age has meant that most experiments have been conducted on neonatal piglets, often 1 day old. Weaning of piglets, e.g. at 4 weeks old, causes a noticeable increase in the proportion of immature crypt cells within the intestine (Hampson, 1986). Conventional neonatal and weaned pigs are markedly resistant to infection with Campylobacter spp (McCartney, 1982; Chang et al, 1984b), therefore some form of barrier rearing would probably be essential for any successful experiment.

Certain breeds of hamsters, particularly the long-haired type, are thought to be more susceptible to proliferative enteritis (Jacoby et al, 1975; Battles, 1985).

The influence of diet on the development of proliferative enteritis in hamsters was investigated by Jacoby and Johnson (1981). Certain diets of unspecified composition could block the effect of known infectious material. Speculative reasons for this could be the introduction of competing dietary antigens capable of altering the intestinal immunity (Stokes et al, 1983), or disturbance to crypt cell metabolism by alterations in dietary fibre content. A similar dietary effect on infection was observed in studies on murine colonic hyperplasia due to Citrobacter freundii (Barthold et al, 1977).

As with most bacteria, Campylobacter sp can rapidly lose virulence

if subcultured (Tucker and Roberstad, 1956; Firehammer and Hawkins, 1964). Bacterial inocula must possess a full set of appropriate cellular and outer membrane components to be virulent, some of which may disappear on subculture.

The possible requirement of a primary or "starter" agent (e.g. a virus) to alter crypt cells such that Campylobacter sp may enter them and be associated with proliferative enteritis has been suggested (Boosinger et al, 1985; Mapother et al, 1987a; Joens et al, 1987). However previous experiments by McCartney et al (1984) established that infections of piglets with rotavirus, Escherichia coli or Cryptosporidia sp did not enhance the ability of C.mucosalis to readily enter piglet ileal enterocytes. Indirect evidence of viral involvement in some lesions of proliferative enteritis was suggested in recent experiments (Mapother et al, 1987a; Joens et al, 1987). Piglets dosed with a 0.22µm filtrate of homogenates of proliferative enteritis mucosae subsequently developed a non-specific enteritis. Rotavirus and enterovirus particles were identified in some of these homogenates. A reduced immune response may have been involved in the development of both proliferative enteritis and cryptosporidiosis in a hamster (Davis and Jenkins, 1986).

Tissue Culture of Campylobacter sp

The difficulty in correlating the culture of one or other Campylobacter spp with the presence of lesions and the experimental reproduction of proliferative enteritis, may mean that the intracellular bacteria characteristic of the disease are obligate intracellular parasites which require an appropriate tissue cell culture for in vitro propagation and/or expression of virulence determinants.

The biology of parasitic intracellular bacteria was reviewed by Formal et al (1983) and Moulder (1985). Briefly, the major enteropathogens in this group initially attach to host enterocytes by recognition of complementary enterocyte receptors, with a sugar moiety at the active site, e.g. C.jejuni can attach to L-fucose (Cinco et al, 1984; McSweegan and Walker, 1986). However the subsequent mechanisms of entry into cells and the means of avoiding or resisting phagosomes vary greatly between bacterial genera. These mechanisms are not fully established for the enteropathogens, but some Rickettsia spp, like the Campylobacter-like organism in proliferative enteritis, lie free in the cytoplasm of parasitised cells. Rickettsia possess phospholipase A which can lyse cell membranes, thus enabling entry into cells, and also lysis of phagosome vacuoles shortly thereafter (Winkler and Miller, 1982). The Rickettsia subsequently multiply free in the cytoplasm (Ewing et al, 1978), in a manner similar to that observed for those bacteria within enterocytes in proliferative enteritis (Lawson et al, 1976; Frisk and Wagner, 1977). The trigger for multiplication of parasitic bacteria is thought to be the concentrations of calcium, potassium, sodium and magnesium that are specific for intracellular fluid (Brubaker, 1967; Perry and Brubaker, 1983). Campylobacter organisms living within enterocytes would have had to develop a method of avoiding or resisting lysosome damage. Several such methods have been developed by parasitic intracellular bacteria (see Moulder, 1985).

As much of the biology of the Campylobacter-like organisms within enterocytes in proliferative enteritis is not known, cell culture models have been studied (Jacoby et al, 1978; Rajasekhar, 1981; Jacoby and Johnson, 1981). Principles for the use of cell culture models to study intracellular bacteria were outlined by Devenish and Schiemann (1981). The initial infection phase must limit the number of bacteria

applied to the cell culture to less than the maximum which can be accommodated by the animal cell without disruption, and the intracellular growth phase must include a provision for blocking continual infection by extracellular bacteria. This blocking is usually accomplished by adding antibiotics or specific antiserum to the medium, or by repeated washing of the cell monolayer. Problems with cell culture models are that they do not match the architectural or functional complexity of natural tissues and that an artificially high dose of bacteria is often used (Moulder, 1985).

However their use may be justified as an initial screen of bacterial invasiveness, and for study of parasitic relationships of bacteria to cells (Formal et al, 1983).

Rajasekhar (1981) established that C.mucosalis could enter and multiply within several continuous epithelial cell lines, particularly bovine or porcine kidney cells. Okereke (1985) further showed that Campylobacter-like bacteria purified from lesions of porcine proliferative enteritis could enter porcine kidney cells. However such bacteria purified from lesions of haemorrhagic enteropathy did not enter or multiply within these cells. Where cell infection by bacteria occurred, it was associated with degeneration of parasitised cells after 10 days of incubation. Particulate matter within parasitised cells bound to specific antiserum prepared against bacteria purified from lesions of porcine proliferative enteritis, in an immunofluorescence assay. Similar binding was also detected between this antiserum and particles within kidney cells infected by C.mucosalis.

Similarly, bacteria purified from lesions of proliferative enteritis in the hamster could enter and multiply within CER (chicken/hamster) or mouse liver cells, and to a lesser extent, within

primary hamster embryo cells (Jacoby et al, 1978; Jacoby and Johnson, 1981). Intracellular bacteria present within these cells one week after infection also bound to convalescent hamster serum, but not to normal rabbit serum or hyperimmune serum raised against various intestinal bacteria (Campylobacter spp were not tested). In the second week after infection, intracellular bacteria started to degenerate. Hamsters dosed with bacteria grown for one week in these cells did not develop proliferative lesions. These models illustrate that intracellular bacterial antigen present in natural proliferative enteritis (Jacoby and Johnson, 1981; Lawson et al, 1985) can be detected in cultured cells.

In both porcine and hamster cell culture models, cells infected with intracytoplasmic bacteria could not be passaged more than once, despite weekly attempts, and no hyperplasia was observed in infected cell lines. These failures may be due to the infected cells not being the target cells of proliferative enteritis. However hamster enterocytes were reported to be difficult to cultivate in vitro (Trier, 1976) and no studies of pig enterocytes have been reported.

The mechanism whereby bacteria within enterocytes could trigger crypt cell hyperplasia is not known, and no comparable model exists. Cell cycle models suggest that hyperplasia is usually due to an increased rate of production of undifferentiated cells, and a quicker rate of cell division; cell differentiation and division occurring at two separate phases of the cell cycle (Scott et al, 1982). The normal rodent villus-crypt unit is approximately 110 cells high, the bottom 22 cells being proliferative crypt cells, and the next 14 cells maturing crypt cells. Only 12 hours is required for a cell to move from the bottom to the top of a crypt. Thus there is a "critical decision zone" wherein crypt cells are moderated to mature or to re-enter DNA

synthesis. This zone is locally regulated by the number of mature enterocytes in the villus above, if these are depleted then an extra division of crypt cells occurs within that unit after a period of 8 hours (Galjaard et al, 1972; Rijke et al, 1976). Mature enterocytes produce a high molecular weight hormone which specifically inhibits crypt cell differentiation and DNA synthesis (Quaroni and May, 1980). However direct loss of villus cells is not a feature of proliferative enteritis (Jacoby, 1978; Rowland and Lawson, 1986). Thus other mechanisms are probably involved.

Firstly the bacteria infecting crypt cells could produce or cause the host cells to produce mitogens or other factors which reduce cell differentiation. The intracellular parasite Chlamydia sp can synthesise lipids or proteins capable of altering the function of host cells (Moulder, 1985). Intracellular, non-membrane bound Rickettsia sp can induce significant organelle changes resulting in cell hypertrophy without gross cell damage. The toxin or other possible mechanism for this effect is not known (Ewing et al, 1978). Certain strains of Citrobacter freundii can attach to the colonic mucosa of mice and produce uncharacterised toxic factors which reduce differentiation of underlying crypt cells (Barthold et al, 1978). Some viruses can produce transforming growth factors which enhance the DNA replication of host cells (Sassone-Corsi et al, 1985). Therefore the intracellular bacteria in proliferative enteritis may be involved in the production of similar growth factors. These factors are thought to combine with cell receptors and activate oncogenes (Goustin et al, 1986). Alternatively the intracellular bacteria may produce toxins which inhibit the enzymes, hormones and other substances thought to induce the normal differentiation of crypt cells (Quaroni and May, 1980).

Secondly part of the immunological response to the bacteria could

include the release of factors which induce crypt cell hyperplasia. T-lymphocytes involved in helminthic and protozoal infections of the intestine can produce factors which enhance crypt cell production, above the enhancement due to direct loss of villus cells in these infections (MacDonald and Ferguson, 1978; Befus and Beinenstock, 1982). However lymphocytic infiltration is not a noticeable feature of the early stages of proliferative enteritis (Jacoby, 1978) and lymphocytic factors capable of inducing proliferation of cells other than lymphocytes are poorly defined.

Analysis of Intracellular Bacteria within Enterocytes in Lesions of Proliferative Enteritis

The notable failure to identify the aetiology of proliferative enteritis warrants analysis of organisms within lesions.

Immunological staining

Sections of tissues affected by proliferative enteritis have been stained by indirect immunofluorescence using polyclonal antisera raised in rabbits against various Campylobacter spp, other enteric organisms and bacteria purified from lesions of proliferative enteritis by filtration (Jacoby and Johnson, 1981; Chang et al, 1984a; Lawson et al, 1985). Formalin fixation of these tissues did not significantly decrease staining of some antigens provided that sections were firstly digested with trypsin (Lawson et al, 1985; Stills et al, 1987). Immunological staining of proliferative enteritis is confused by a component present in the serum of some normal laboratory rabbits which reacts with intracellular bacteria in lesions (Lawson et al, 1985). The aetiology of this component is not known; a speculative source may be natural antibody to a Campylobacter sp infection of the rabbit (Moon et al, 1976). Therefore some of the reactions described in previous

immunofluorescence studies using rabbit antiserum raised against C.mucosalis or C.hyointestinalis (Rowland and Lawson, 1974; Chang et al, 1984a) may not have been specific.

Reliable staining of bacteria within affected enterocytes only occurs if rabbit antiserum prepared against intracellular bacteria purified from lesions, or convalescent serum is used (Jacoby and Johnson, 1981; Lawson et al, 1985). These sera did not react with pure cultures of any Campylobacter sp tested. Recently, a monoclonal antibody showing general reactivity to many Campylobacter spp also reacted with intracellular bacteria in lesions of proliferative enteritis in the hamster (Stills et al, 1987). This may indicate that these bacteria are indeed a form of Campylobacter sp.

The reason for the apparent antigenic exclusivity of the intracellular bacteria is not clear. It may be that the organism is an intracellular parasite which has not yet been cultured, even though it closely resembles Campylobacter sp.

Alternatively the organism could alter its antigenic coat during entry into the enterocyte. Outer membrane fragments are frequently released during the growth of Gram-negative bacteria, including Campylobacter sp (Logan and Trust, 1982). These fragments are usually rich in newly synthesised proteins, lipopolysaccharides and periplasmic proteins, thus they can present a different antigenic profile to that of the intact bacterium (Logan and Trust, 1982). It is possible that the entry into the cell and production of such fragments is linked. Another possibility is that the bacterial membrane may be altered by the acquisition or induction of special enzymes or receptors (see below).

The ability of intracellular enteropathogenic bacteria to express those membrane proteins necessary for attachment, entry and

multiplication into host cells is often determined by specific plasmids (Hale et al, 1983; Portnoy et al, 1984). Plasmid-encoded membrane polypeptides from different enteropathogens are a relatively homogenous group, indicating a functional and evolutionary relationship (Hale et al, 1983; Formal et al, 1983). Approximately 70 per cent of porcine isolates of C.jejuni/coli carry plasmids (Bradbury and Munroe, 1985). The functional significance of these is not clear, although some may carry antibiotic resistance genes (Taylor et al, 1987).

Study of the chromosomal DNA of Campylobacter sp involved in proliferative enteritis has been limited to taxonomic comparisons of known strains (Lawson et al, 1981; Gebhart et al, 1985a).

Antigens of Campylobacter sp

Monoclonal antibodies - Production of these antibodies through fusion of myeloma cells and lymphocytes is now a well-established technique (Kohler, 1981). As a monoclonal antibody only reacts with one antigenic site, their reactivity depends on the distribution of this site (= epitope) within a group of bacteria. In practice, most monoclonal antibodies prepared against a bacterial culture only react with one strain or a few strains of that bacterium's species, however a few will react with a variety of other bacteria. Therefore the technique can be used to study the distribution of antigens in a species or to produce a monospecific antiserum. Monoclonal antibodies have been prepared against the intracellular bacterium, Mycobacterium leprae extracted from infected armadillo liver. Most were directed against specific membrane protein and glycoprotein components, but some showed cross reactions with other Mycobacteria spp (Gillis and Buchanan, 1982; Kolk et al, 1984; Engers et al, 1985). Some showed cross-reactions with non-infected armadillo liver in both

immunofluorescence and enzyme immunoassays (Engers et al, 1985). Similarly, monoclonal antibodies prepared against Treponoma pallidum extracted from rabbit testicles, showed considerably fewer cross-reactions with testicle tissue, if the T.pallidum were carefully extracted first (Lukehart, 1986).

Kosunen et al (1984) developed 29 monoclonal antibodies to whole C.jejuni from one mouse fusion. Some of these showed cross-reactions with C.fetus or C.coli in an enzyme immunoassay, but none reacted with unrelated bacteria. Monoclonal antibodies prepared against purified flagella from C.jejuni showed significant reactivity with antigenic sites on the surface of whole C.jejuni (Nachamkin and Hart, 1986; Newell, 1986a,b). A C.jejuni/coli strain isolated from a hamster with proliferative enteritis had identical flagellar antigens to those of a human strain (Nachamkin and Hart, 1986).

Monoclonal antibodies against Campylobacter spp have mostly been used as a tool for serology (Kosunen and Hurme, 1986), but a recent study used them to try to identify the intracellular Campylobacter-like organisms in proliferative enteritis in hamsters (Stills et al, 1987). It was found that these organisms did not react with a C.jejuni/coli specific antibody, but did react with an antibody showing reactivity to many Campylobacter sp, in an immunofluorescence assay.

Surface Protein Antigens - Separation of bacterial proteins by treatment with sodium dodecyl sulphate and electrophoresis in polyacrylamide gels is a well-established technique (Ames, 1974). Most Campylobacter sp show a distinct protein profile, but each species probably has the following similar major proteins: transmembrane porin protein, flagellin, and an acid-dissociable protein (Logan and Trust, 1983; Hanna et al, 1984; Wenman et al, 1985). For C.jejuni/coli the molecular weights of these are 44K, 62K and 30K, respectively.

Electrical transfer of these proteins onto nitrocellulose sheets allows various antisera to be reacted directly with the separate proteins (immunoblotting). Comparison of the blots obtained with acute and convalescent antisera from an infected animal will identify those antigenic proteins of the bacterium that are actually recognised during the infection (Burnette, 1981). Human infection with C.jejuni provokes a specific serum and intestinal antibody response to the three major proteins just mentioned, and to several other minor proteins (Logan and Trust, 1983; Mills and Bradbury, 1984; Blaser et al, 1984, 1986). This response is specific, there being little response in the serum of C.jejuni patients to C.fetus antigens (Blaser et al, 1984). Studies of the surface proteins of C.mucosalis and C.hyointestinalis have not been published.

The surface proteins of C.jejuni may show significant antigenic variations between pathogenic (enterotoxic) and non-pathogenic strains (Klipstein et al, 1985, 1986). However this conclusion was based only on enzyme immunoassay results, and the ability to clearly recognise the pathogenicity of any strain by in vitro tests is still at an uncertain stage. Therefore, it is unreliable to make further conclusions about differences in bacterial proteins between strains of Campylobacter spp. Significant antigenic variations between pathogenic and non-pathogenic strains have been detected in gel electrophoresis patterns of other Gram-negative bacteria such as Neisseria gonorrhoeae (Lambden et al, 1979). These variations are due to the expression of surface proteins or glycoproteins in pathogenic strains, which can influence their resistance to serum factors (Lambden et al, 1979; Munn et al, 1982). Similar functional antigenic variation was recently demonstrated in C.fetus (Blaser et al, 1987) where an outer membrane protein of 100K was thought to mediate serum resistance. The depletion of iron salts

in the environment of bacteria can result in alterations of their antigenic protein profile (Hantke, 1985). In vitro tests with C.jejuni confirmed that these alterations were consistent with induction of membrane iron receptors, called siderophores and the production of iron-chelating compounds by the bacteria (Field et al, 1986). In vivo tests with Vibrio cholerae suggested that these receptors were correlated positively with strain virulence (Sciortino and Finkelstein, 1983), however this correlation has not been reported elsewhere.

Gel electrophoresis and immunoblotting have been used to probe the relationship of the non-culturable bacterium Treponema pallidum to culturable treponemes (Lukehart et al, 1982).

Outer Membrane Lipopolysaccharides - The outer membrane lipopolysaccharides (LPS) of Campylobacter spp consist of a core lipopolysaccharide unit, with carbohydrate side-chains of varying length and a lipid-A or toxic moiety. Bacteria with long side-chains (smooth) are often resistant to serum bactericidal activity and therefore capable of causing septicaemia, e.g. C.fetus; whereas bacteria with short or no side-chains (rough) are often sensitive to serum and associated with local or enteric infection, e.g. C.jejuni (Perez et al, 1985). Lipopolysaccharide side-chains of some C.jejuni strains had strong adhesin reactions with intestinal cell receptors (McSweeney and Walker, 1986). Intracellular bacteria with high LPS content, e.g. Brucella sp can have an increased ability to survive host cell phagosome enzymes (Riley and Robertson, 1984).

Strains of C.fetus and C.jejuni vary greatly in their LPS profiles on gel electrophoresis systems, i.e. these profiles can not be defined for each species (Perez and Blaser, 1984; Perez et al, 1985). These variations are due to minor changes in the nature of the side-chains (Hitchcock and Brown, 1983), and influence agglutination reactions (see

below).

A preliminary study indicated that a LPS fraction extracted from C.mucosalis shared antigen-antibody reactions with a LPS fraction of bacteria purified from lesions of proliferative enteritis in complement fixation tests, (Lawson and Rowland, 1984). However the heterogeneity of LPS in Campylobacter spp does not allow any definite conclusions to be drawn from this result.

Small carbohydrate units within bacterial LPS can react with lectins. These are proteins or glycoproteins of non-immune origin (often plant seeds) with binding specificities for carbohydrates. Various Campylobacter spp strains have been shown to have specific lectin reaction patterns (Wong et al, 1985; Gill and Corbel, 1986).

Serology

Agglutination reactions in slides, tubes or wells between Campylobacter sp antigen and sera from affected animals or immunized rabbits have suggested that C.mucosalis has three serotypes (A, B, C) (Lawson et al, 1977) and that C.hyointestinalis has two to four serogroups (Terzolo, 1984). Agglutination reactions of C.jejuni are now considered to be due to bacterial LPS-serum reactions (Mills et al, 1985). Serum from rabbits immunized with bacteria purified from affected tissue did not agglutinate either C.mucosalis or C.hyointestinalis antigen (Lawson et al, 1985). Therefore serology has not yet helped to identify the intracellular bacteria in proliferative enteritis.

The serum antibody reactions to human enteric infections with C.jejuni were clarified by enzyme immunoassay of acute and convalescent sera (Blaser and Duncan, 1984). Persons with C.jejuni-associated enteritis developed rising IgA, IgG and IgM concentrations in their

serum by the second week after infection. IgG and IgM elevations persisted longer than those of IgA. Raw milk drinkers and slaughterhouse workers, i.e. chronically exposed persons, had chronically elevated serum IgG concentrations. The 30K, acid-dissociable outer membrane protein of C.jejuni is a major "shared" antigen, as it incites a significant specific reaction in the serum of all patients infected with C.jejuni, regardless of the strain involved (Blaser and Duncan, 1984). The enzyme immunoassay reactions of other Campylobacter spp involved in proliferative enteritis have not been reported.

Further Research

The major unanswered problem in proliferative enteropathy is still the aetiology and pathogenesis of the disease. This problem could be resolved if the identity and role of the intracellular Campylobacter-like organisms were known. A key experiment would be the experimental reproduction of the disease with a pure culture or suspension of an identifiable agent. The failure of previous studies suggests that simple culture/inoculation experiments would be unlikely to yield results. Therefore I propose to conduct experimental transmission studies in hamsters and pigs with the aim of reproducing the disease, but taking into account the virulence of inocula and host immunity and diet. Further insight into the identity of the intracellular organisms may be gained by comparison of their antigens to those of cultured Campylobacter spp. Similarity between their antigens and those of one species would naturally indicate a relationship. Therefore I propose to investigate the purification of intracellular organisms by biochemical separation techniques such as affinity chromatography. Immunological comparisons may then be instigated, such as use of

monoclonal antibodies, immunoblotting and related techniques.

CHAPTER 2

ORAL INOCULATION OF GNOTOBIOTIC PIGS WITH INTESTINAL
MUCOSA FROM PIGS AFFECTED BY PROLIFERATIVE ENTEROPATHY

Introduction

Of numerous experiments designed to reproduce porcine proliferative enteritis prior to the date of this study (1986), probably only two succeeded (Roberts et al, 1977; Lomax et al, 1982b). In both, conventional pigs were dosed orally with fresh intestinal mucosa from naturally-affected pigs. Lesions were noted at autopsy three to eight weeks later. Although Campylobacter mucosalis was isolated from "successful" tissue inocula in those experiments, cultures of C. mucosalis alone have uniformly failed to cause proliferative enteritis, with or without significant intestinal colonisation of orally dosed gnotobiotic piglets (McCartney et al, 1984; Chang et al, 1984b; Boosinger et al, 1985). Similarly several authors have tested infection with C. hyointestinalis and C. coli, which may be isolated from naturally occurring porcine proliferative enteritis and have failed to reproduce the disease, although these organisms can colonise the porcine intestine for long periods (Davis, 1961; Andress et al, 1968; Kashiwazaki et al, 1971; Olubunmi and Taylor, 1982; Chang et al 1984b; Boosinger et al, 1985).

The failure of Campylobacter sp strains alone to reproduce the disease means it is possible that strains lost virulence during storage, handling or passage on artificial media, that dosed pigs were somehow resistant or that the wrong organism was used. To circumvent such problems, gnotobiotic piglets were used in this study, and dosed with crude intestinal inocula known to contain different Campylobacter sp. To lessen the obvious contamination of such inocula with other agents likely to be harmful to gnotobiotic animals, the inocula were selectively filtered for organisms the size of Campylobacter sp, prior to dosing.

Materials and Methods

Piglets

Neonatal gnotobiotic piglets were derived by methods routinely used at the Institute for Animal Disease Research, Compton, Berkshire (Tavernor et al, 1971; Trexler, 1971). Briefly, an adult sow (Sus scrofa domestica) in late pregnancy was anaesthetised, and prepared for aseptic abdominal surgery. A left flank incision was made from the inside of a sterile plastic "bubble" and eight piglets removed from the uterus. After cleaning, resuscitation and navel disinfection, pairs of piglets were introduced, via a sterile transport "bubble" into four isolators (Fig 1).

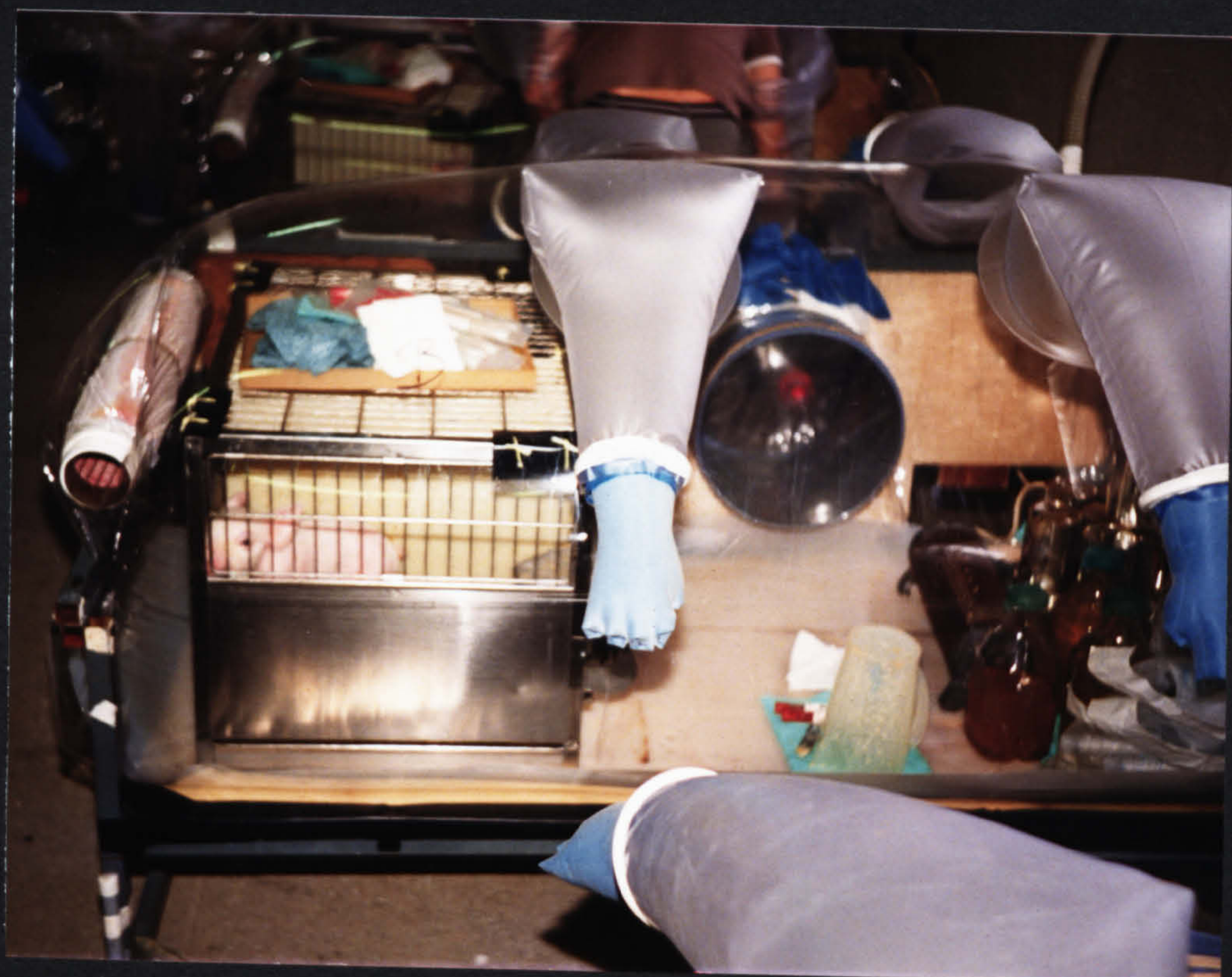
Pigs were fed a monitored amount of a proprietary sterilised evaporated milk preparation supplemented with a vitamin/mineral mixture thrice daily. Each isolator's temperature and humidity were controlled to 30 to 37°C and 40 to 70 per cent respectively. All isolation and surgical equipment was sterilised prior to use by autoclaving, or spraying with 2 per cent v/v peracetic acid where appropriate. Oral and rectal swabs were taken at least twice weekly throughout and processed for bacteriological culture. All swabs taken prior to inoculation of the piglets proved sterile when cultured.

Inocula

Four inocula were prepared from three porcine intestines affected by histologically confirmed proliferative enteritis. The mucosae had been collected fresh into sterile vials, and stored frozen at -70°C for two months. For each inoculum, 10g of mucosa was washed twice in Dulbecco's modification of Eagles's medium (Gibco, U.K.), and mixed with 20ml of 1 per cent trypsin in phosphate-buffered saline, pH 7.4 (PBS, see Appendix 1) for 35 minutes at 37°C. After homogenisation for

FIGURE 1.

Plastic isolater containing a pair of gnotobiotic piglets,
six days old, (pigs 3 and 4), at Institute for Animal
Disease Research, Compton, Berkshire.



30 seconds in a blender (M.S.E. Instruments, U.K.), the resulting suspension was diluted with 20ml of the above medium, and filtered sequentially by positive pressure through 47mm cellulose acetate filters (Millipore, U.K.) of 2.5 μ m, 1.5 μ m and 0.8 μ m pore diameter. Three of the filtrates were further passed through 0.65 μ m filters (see Table 1). The filtrates were introduced aseptically into the isolators, and the piglets each dosed with 19ml of filtrate introduced orally via a syringe and tube. One millilitre of each inoculum was removed from the isolators and standard dilutions made in phosphate-buffered saline for colony counting (Miles and Misra, 1938). Smears of each filtrate were stained by modified acid-fast method (see Appendix 3). Cultures from each dilution were incubated at 37°C on horse blood agar, Skirrow's agar (Skirrow, 1977) and RNBGT agar (McCartney et al, 1984) in an atmosphere of 3 to 6 per cent oxygen, 10 per cent carbon dioxide, and 70 per cent hydrogen, with nitrogen. Campylobacter spp isolates were identified by biochemical and slide agglutination reactions (Lawson et al, 1976, 1985; Hebert et al, 1982; Gebhart et al, 1985a). Bacterial isolates other than Campylobacter spp were partially identified by standard tests (Cowan, 1974). Oral swabs were inoculated onto blood agar plates. Rectal swabs were assumed to contain 0.2g of faeces and dilutions for colony counting were prepared in phosphate-buffered saline. Cultural procedures were as described above.

Necropsy Procedures

Pathology - Pigs were anaesthetised with intravenous pentobarbitone and killed by exsanguination. Portions of intestine and other tissues were collected and fixed in buffered formalin. Histological sections of 5 μ m were prepared and stained by haematoxylin and eosin, and a silver impregnation stain (Young, 1969).

TABLE 1: Experimental design and inocula

Pig	Inocula No. and diagnosis	Final filter (µm pore size)	Estimated number of <u>Campylobacter</u> sp cultured (numbers expressed as log ₁₀ ml ⁻¹)	<u>Campylobacter</u> -like organisms in smears ^a	Age at dosing (days)	Age at necropsy (days)
1	284/86 PHE	0.8	<u>C.coli</u> 3.5	+++	7	17
2	284/86 PHE	0.8	<u>C.coli</u> 3.5	+++	7	27
3	284/86 PHE	0.65	<u>C.coli</u> 2.4	++	7	17
4	284/86 PHE	0.65	<u>C.coli</u> 2.4	++	7	28
5	716/86 PHE	0.65	<u>C.mucosalis</u> 4.5 <u>C.coli</u> 6.4	+++	12	22
6	716/86 PHE	0.65	<u>C.mucosalis</u> 4.5 <u>C.coli</u> 6.4	+++	12	30
7	761/86 PIA	0.65	<u>C.mucosalis</u> 5.4 <u>C.coli</u> 4.8	+++	12	13
8	761/86 PIA	0.65	<u>C.mucosalis</u> 5.4 <u>C.coli</u> 4.8	+++	12	21

PHE Proliferative haemorrhagic enteropathy

PIA Porcine intestinal adenomatosis

a ++ 5-10, +++ >10 Campylobacter-like organisms per high power field

Further portions of ileum and proximal colon were collected and fixed in 2.5 per cent glutaraldehyde in phosphate-buffered saline. These portions were post-fixed in osmium tetroxide, embedded in epoxy resin and 50nm sections were cut and placed on copper grids. Sections were stained with saturated uranyl acetate in 5 per cent ethanol (30 minutes) then Reynold's lead citrate solution (5 minutes) and examined with an electron microscope (Philips EM400). Further thin sections were cut, stained with a silver impregnation stain (Young, 1969) modified by development at 37°C, and examined histologically.

Bacteriology - The stomach (fundic zone), duodenum, jejunum, ileum, caecum, proximal and spiral colon were opened aseptically and one gram of mucosa from each site scraped into 9ml of sterile phosphate-buffered saline and homogenised. Cultures and viable counts were made on each homogenate as described above. Oral and rectal swabs and swabs of ileal and colon contents taken at autopsy were similarly processed.

Indirect Immunofluorescence - Unstained sections of formalin-fixed intestine from each pig were digested in trypsin and prepared for immunofluorescence as described previously (Lawson et al, 1985). Antisera prepared in rabbits and guinea-pigs to Campylobacter-like organisms purified from lesions of proliferative enteritis in pigs 1269/76 (Lawson et al, 1985) and 284/86 (Table 1) respectively, antisera prepared in both rabbits and guinea-pigs to two strains of C.hyointestinalis and C.coli from pigs 1 and 2 (Table 1), and appropriate fluorescein-conjugated anti-species immunoglobulin (Nordic Laboratories, U.K.) were used in indirect tests. These strains of C.hyointestinalis were designated 9AL2 and 9AL3, and C.coli strains 9AF3a and 9BF2. The C.hyointestinalis strains were isolated from the large intestine of pig 1 at necropsy, the C.coli strains from the

faeces of pigs 1 and 2 on days 8 and 2 post-inoculation respectively.

Antisera was produced by schedules previously described for rabbits (Lawson and Rowland, 1974). Briefly, adult New Zealand white rabbits were intravenously inoculated with bacterial suspensions of 10^8 cells per millilitre from 24h plate cultures. Cultures were harvested in phosphate-buffered saline, pH 7.4 (see Appendix 1) with 0.3 per cent v/v formalin and cell count standardized to Brown's opacity tube number 2. Identical booster doses of 0.5ml were given intravenously on days 4, 7 and 10 after the initial immunization. On day 20 the rabbits were bled and the sera were collected and stored at -20°C . Pre-immune sera and serum from a rabbit immunised with an unrelated organism, a laboratory strain of "Haemophilus sommus" 578/77, were used as controls. Separate sections were stained with each antiserum at a working dilution obtained from preliminary immunofluorescence assays with homologous antigen, see Chapter 4. Sections from a normal pig designated 42/78 were also stained with each antiserum.

Guinea-pigs were injected intramuscularly with 0.5ml of antigen, three times at 2 weekly intervals. The first injection contained 0.5ml of antigen emulsified with 0.5ml of Freund's incomplete adjuvant. One week after the last injection, the guinea-pigs were bled for serum. Sera from guinea-pigs before immunisation were used as controls.

Results

Pigs remained clinically normal after inoculation with the exception of pig 7 which became depressed and anorexic and died the day after dosing. No specific lesions were detected at its necropsy.

Gross and microscopical findings

No gross changes were seen at necropsy in any of the pigs. The small intestine contained semi-solid, yellow fluid. The caecum and

colon were filled with a thick yellow paste.

The ileal mucosa of pigs 1 and 2 had numerous enlarged glands, lined by immature basophilic columnar enterocytes (Fig 2). Numerous mitotic figures and reduced numbers of goblet cells were evident at all levels of affected glands. Individual necrotic enterocytes were evident within the lining of affected glands. Similar changes were diffusely evident in all of the large intestinal mucosa of pig 1 and occasionally evident in the mucosa of the spiral colon of pig 3. There was a moderate infiltration of lymphocytes, macrophages and neutrophils in the ileal and large intestinal mucosae and lamina propria (Fig 3), of all pigs. Examination of sections of liver, spleen, mesenteric lymph nodes, stomach and duodenum from each pig showed no significant lesions. Epithelial cells of the jejunum of pig 7 had severe, diffuse vacuolation, particularly of villous cells.

Silver impregnation stains showed that curved bacilli were numerous in the apical cytoplasm of affected enterocytes, as well as in the crypt lumina in the large intestine of pigs 1 and 2 (Fig 4), and in the crypt lumina only in pigs 4, 5, 6 and 8. In pig 1, 70 per cent of large intestinal crypts with enterocytes containing numerous intracellular bacteria, also had numerous luminal curved bacilli. However only occasional curved bacilli were evident in crypt lumina of the small intestines of each pig. No intracellular bacteria were evident in pigs 4 to 8.

No other significant lesions were detected.

Bacteriological findings

Campylobacter spp isolated from the inocula are given in Table 1. The inocula also contained Bacteroides spp, Staphylococcus sp, and other unidentified gram negative bacilli each in concentrations of 2.0

FIGURE 2.

Photomicrograph of section of proximal colon from pig 1
(10 days after infection). Proliferation of immature
enterocytes in crypts, reduction of goblet cells evident.

Haematoxylin and eosin, x 600.

FIGURE 3.

Photomicrograph of section of proximal colon from pig 1.
Several crypts with proliferating enterocytes, with mucosal
infiltration by lymphocytes, macrophages and neutrophils
evident.

Haematoxylin and eosin, x 400.

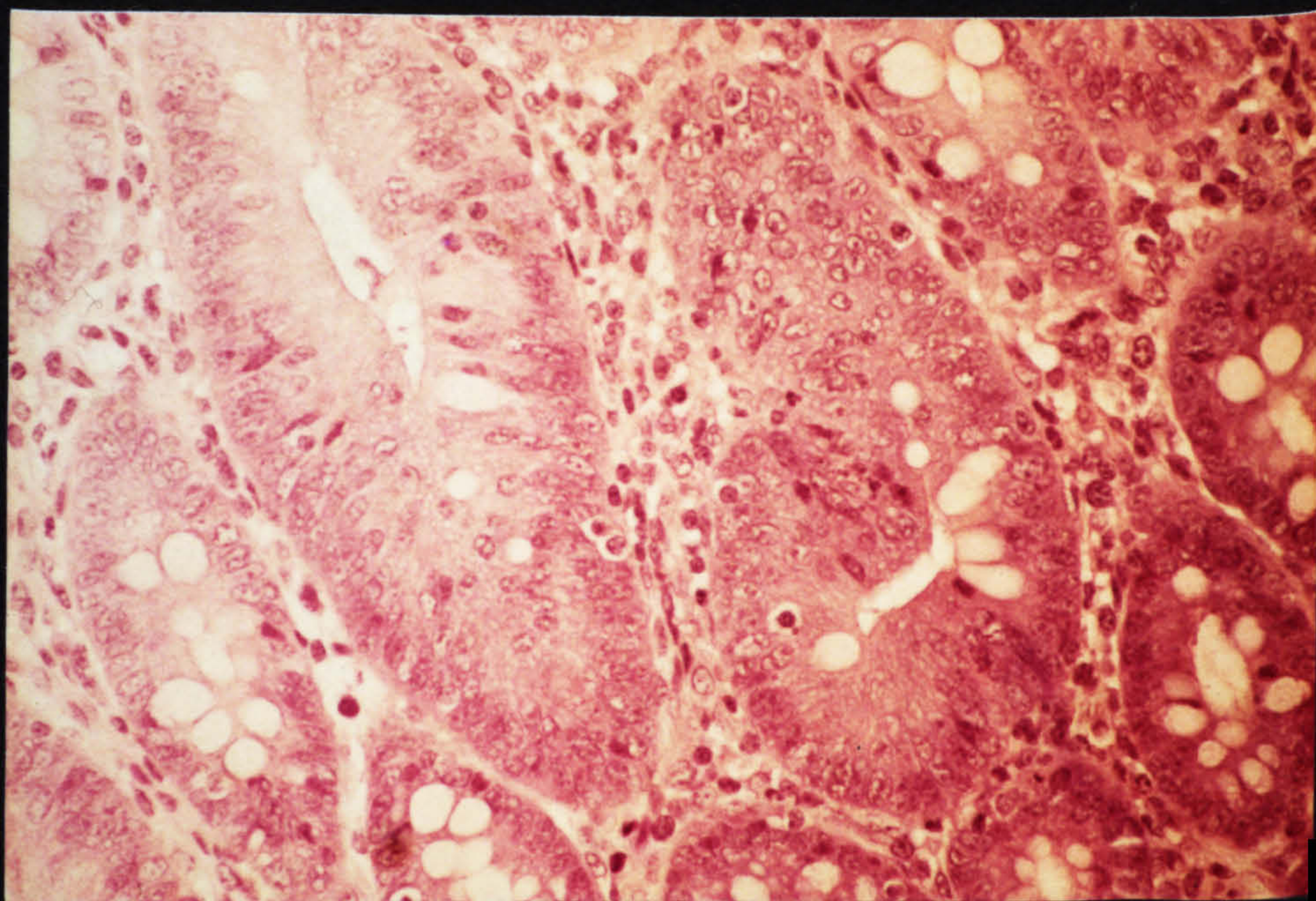
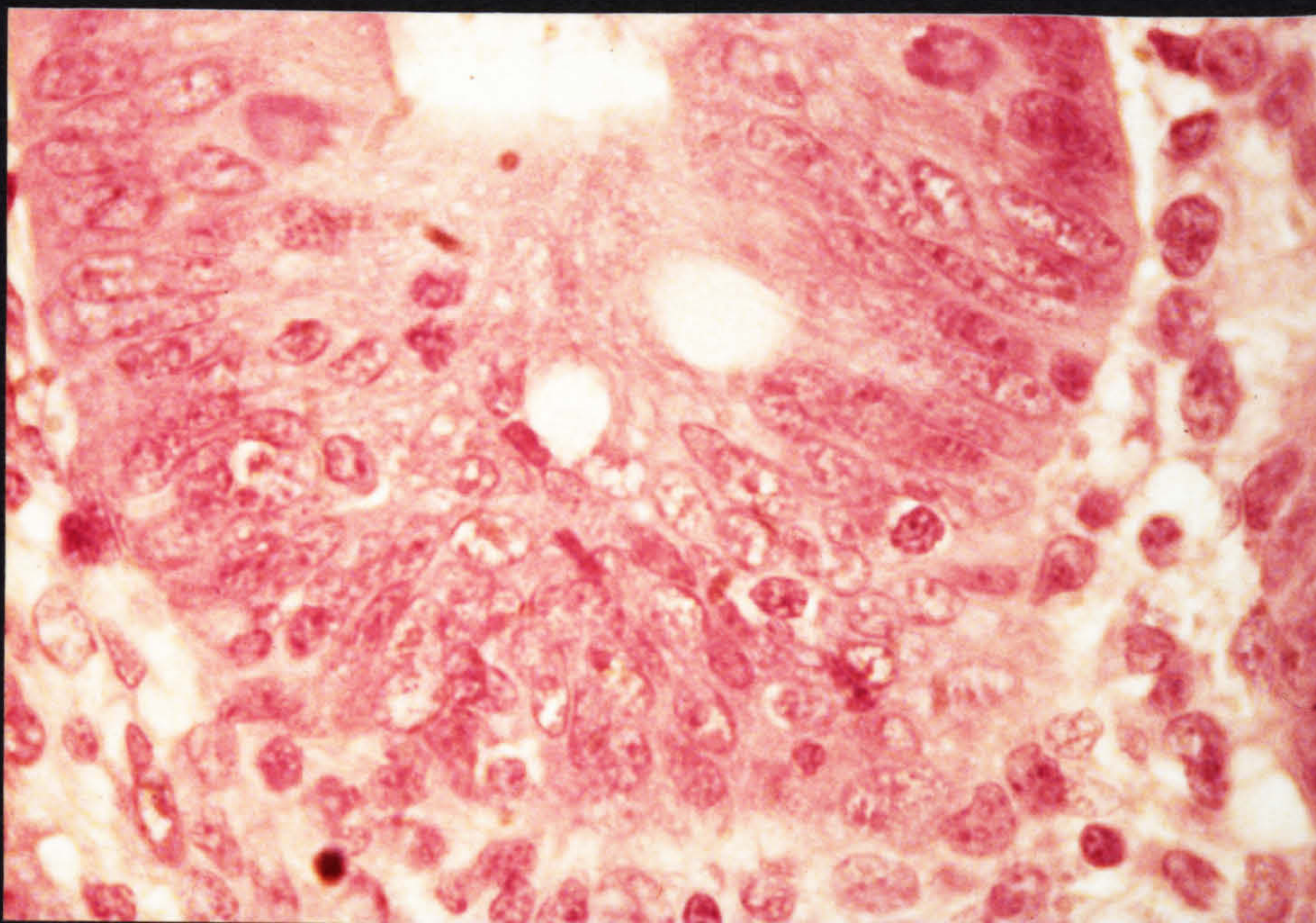
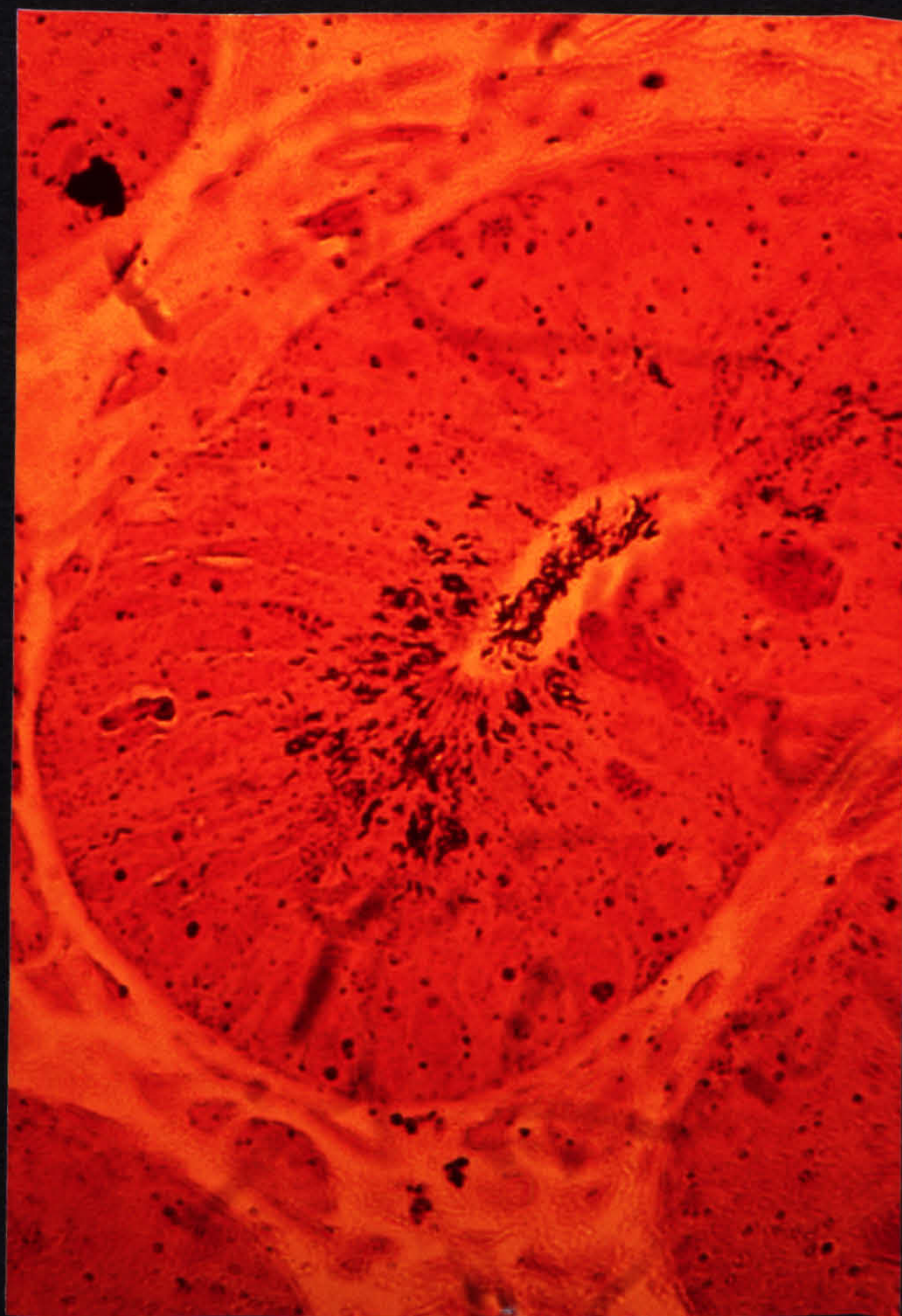


FIGURE 4.

Photomicrograph of thin section (1 μ m) of proximal colon of pig 1. Numerous intracellular Campylobacter-like organisms in proliferating enterocytes, with similar organisms in lumen of crypt.

Silver stain x 600.



to $3.0 \log_{10} \text{ml}^{-1}$.

The numbers of C.hyointestinalis and C.coli in faeces are shown in Table 2. Rectal swabs from all pigs after inoculation consistently contained Bacteroides spp, Staphylococcus sp, and other gram negative bacilli, each at 6.0 to $7.0 \log_{10} \text{g}^{-1}$ faeces. C.mucosalis was isolated from oral swabs taken on days 2 and 5 from pigs 5, 6 and 8, but not from any of the rectal swabs. Table 3 shows the results of cultural examination of the gastrointestinal tracts. C.mucosalis was not isolated from any intestinal site. Bacteroides spp, Staphylococcus sp and other unidentified gram negative bacilli were consistently isolated from intestinal mucosa and contents each in concentrations of 6.0 to $8.0 \log_{10} \text{g}^{-1}$ sample. All samples of gastric and duodenal mucosa were sterile.

Ultrastructural findings

Ileal enterocytes in pigs 1 and 2 and colonic enterocytes in pig 1 showed numerous curved bacilli at various locations in their apical cytoplasm (Fig 5). The microvilli of these cells were poorly developed compared to adjacent non-parasitised cells. The bacteria within these cells did not have an enclosing host cell membrane and were not associated with lysosomes. Occasional dividing forms were evident. Numerous curved bacilli similar to those observed within cells, were found in the associated crypt lumina of the colon of pig 1. Some of these bacteria were closely related to the surface membrane of enterocytes, and some had single large vacuoles in their cytoplasm (Fig 6).

Indirect immunofluorescence

Sections treated with antiserum to intracellular Campylobacter-like organisms showed brightly fluorescing curved bacilli within enterocytes throughout the ileum and large intestine of pig 1 and the

TABLE 2: Estimated number of C.hyointestinalis and C.coli isolated from faeces of pigs dosed orally with filtered inocula (numbers expressed as log₁₀g⁻¹ faeces)

		Days after exposure							
Pig		2	5	8	10	13	15	18	21
1	CHI	NR	4.0	7.0	6.5				
	CC	6.3	6.7	8.0	7.5				
2a	CC	6.3	6.0	7.5	7.0	5.0	5.0	6.0	
3	CHI	NR	4.2	6.3	6.5				
	CC	6.0	7.2	8.4	7.3				
4	CHI	NR	5.0	6.5	6.5	7.3	6.8	6.8	7.0
	CC	6.3	6.0	8.0	7.3	6.0	6.0	6.3	6.5
5a	CC	6.5	7.0	7.3	7.5				
6a	CC	6.5	7.3	7.3	7.8	7.5	7.5	7.8	
8	CHI	NR	4.0	6.3	6.0				
	CC	6.2	6.8	6.5	6.0				

NR C.hyointestinalis not recovered

CHI C.hyointestinalis

CC C.coli

a C.hyointestinalis was not recovered from any sample from these animals.

TABLE 3: Estimated number of Campylobacter spp in samples of intestinal mucosa or contents at necropsy (numbers expressed as log₁₀g⁻¹ sample)

Pig		Jejunum	Ileum	Ileum contents	Large bowel	Large bowel contents
1	CHI	NR	6.5	NR	5.8	6.5
	CC	NR	NR	NR	NR	NR
2a	CC	NR	NR	NR	6.4	6.2
3	CHI	NR	NR	NR	7.2	6.5
	CC	NR	NR	NR	NR	7.2
4	CHI	NR	5.0	5.5	7.0	6.0
	CC●	NR	NR	NR	NR	7.5
5a	CC	7.0	3.5	4.0	8.1	7.2
6a	CC	7.5	6.5	NR	6.8	7.5
7a	CC	NR	NR	NR	NR	6.5
8	CHI	NR	4.0	3.0	5.5	6.0
	CC	NR	NR	3.0	5.8	5.2

NR Not recovered

CHI C.hyointestinalis

CC C.coli

a C.hyointestinalis was not recovered from any samples from these animals

FIGURE 5.

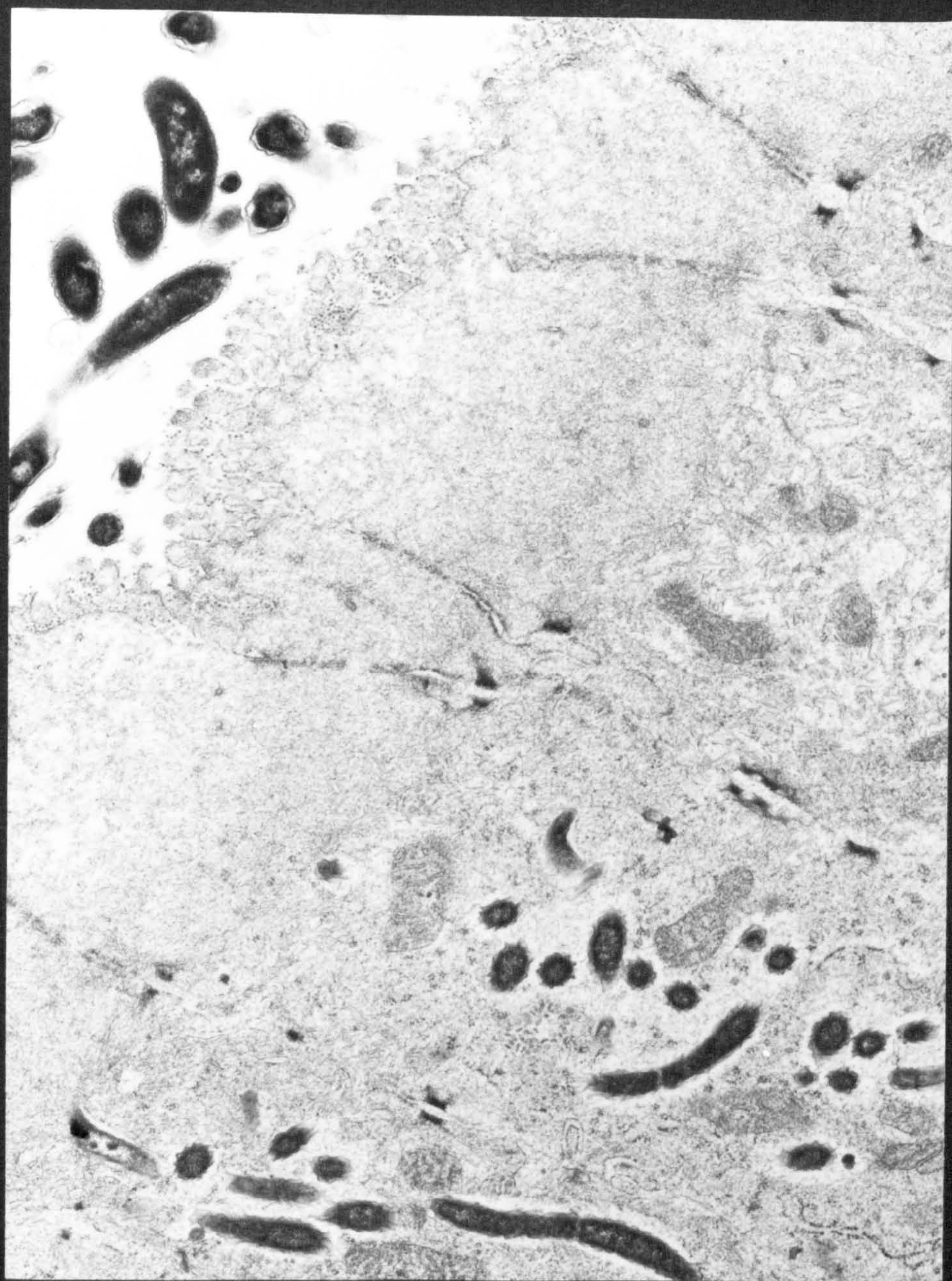
Electron-micrograph of ultra-thin section of ileum of pig 1 (10 days after infection). Several Campylobacter-like organisms within cytoplasm of crypt enterocytes. Reduced numbers of microvilli evident on parasitised enterocytes.

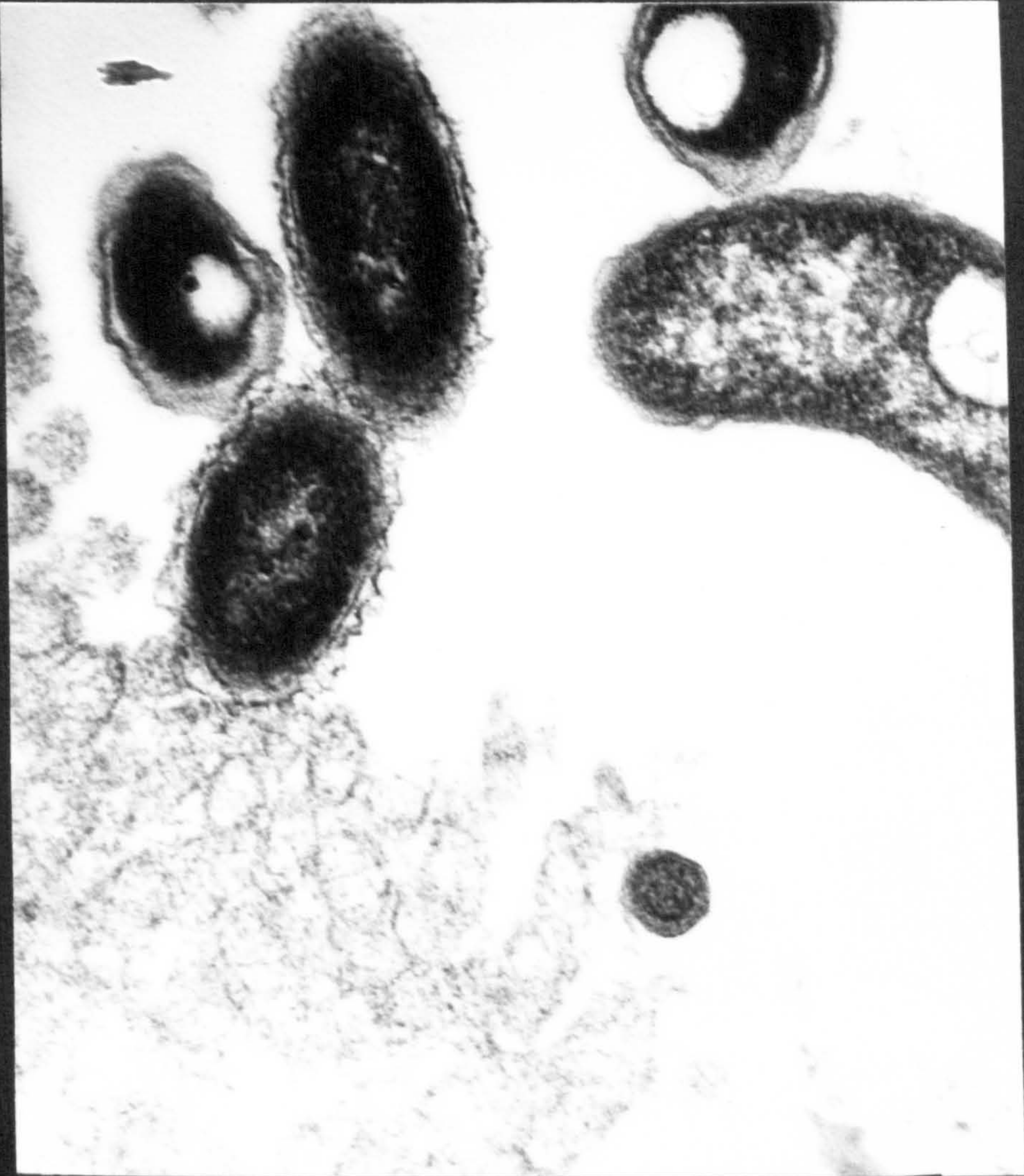
Uranyl acetate/lead citrate stain x 16,500.

FIGURE 6.

Electron-micrograph of ultra-thin section of proximal colon of pig 1. Campylobacter-like organisms associated with brush border of crypt enterocyte. Several of these bacteria contain vacuoles.

Uranyl acetate/lead citrate stain x 66,000.





ileum of pig 2, and within enterocytes in occasional crypts in the spiral colons of pigs 1 and 3 (Fig 7). Intracellular organisms were not demonstrated with this antiserum in sections from other pigs, nor in any pig with the antisera to the cultured Campylobacter spp. Sections treated with antiserum to C.coli showed occasional brightly fluorescing curved bacilli adjacent to the surface epithelium of the large intestine in all of the pigs (Fig 8). These fluorescing bacilli did not correlate with the numerous curved bacilli observed in the lumen of the large intestine of pig 1, in sections stained by silver impregnation. Groups of curved bacilli which fluoresced with antiserum to C.hyointestinalis, in crypt lumina of the large intestine of pig 8, (Fig 9) and in smaller numbers in pig 1, correlated with similar groups observed in the sections stained by silver impregnation. No reactions were observed with serum to "H.somnus" 578/77, with any pre-immune serum, or in normal pig intestinal sections.

Discussion

Two gnotobiotic pigs exposed orally to a filtered suspension of intestinal mucosa from a naturally infected pig subsequently developed lesions of proliferative enteritis. A third such pig developed minor lesions of proliferative enteritis in its spiral colon. Therefore gnotobiotic pigs can be used as an appropriate experimental model for the disease and to some extent, this validates the use of such animals and the previous negative results following oral dosing with pure cultures of C.mucosalis, C.hyointestinalis or C.coli (Andress et al, 1968; Kashiwazaki et al, 1971; Chang et al, 1984b; McCartney et al, 1984; Boosinger et al, 1985; Gebhart, 1987).

The pathogenesis of porcine proliferative enteritis is clearer from the results of this study. Ten days after infection, curved

FIGURE 7.

Photomicrograph of unstained section of ileum of pig 2 (21 days after infection) treated in an indirect immunofluorescence assay. Rabbit antiserum to Campylobacter-like organisms 1269/76, diluted 1:150 in phosphate-buffered saline, was the first antibody, and fluorescein-conjugated sheep anti-rabbit immunoglobulins the second antibody. Brightly fluorescing curved bacilli evident within the cytoplasm of enterocytes.

x 600.

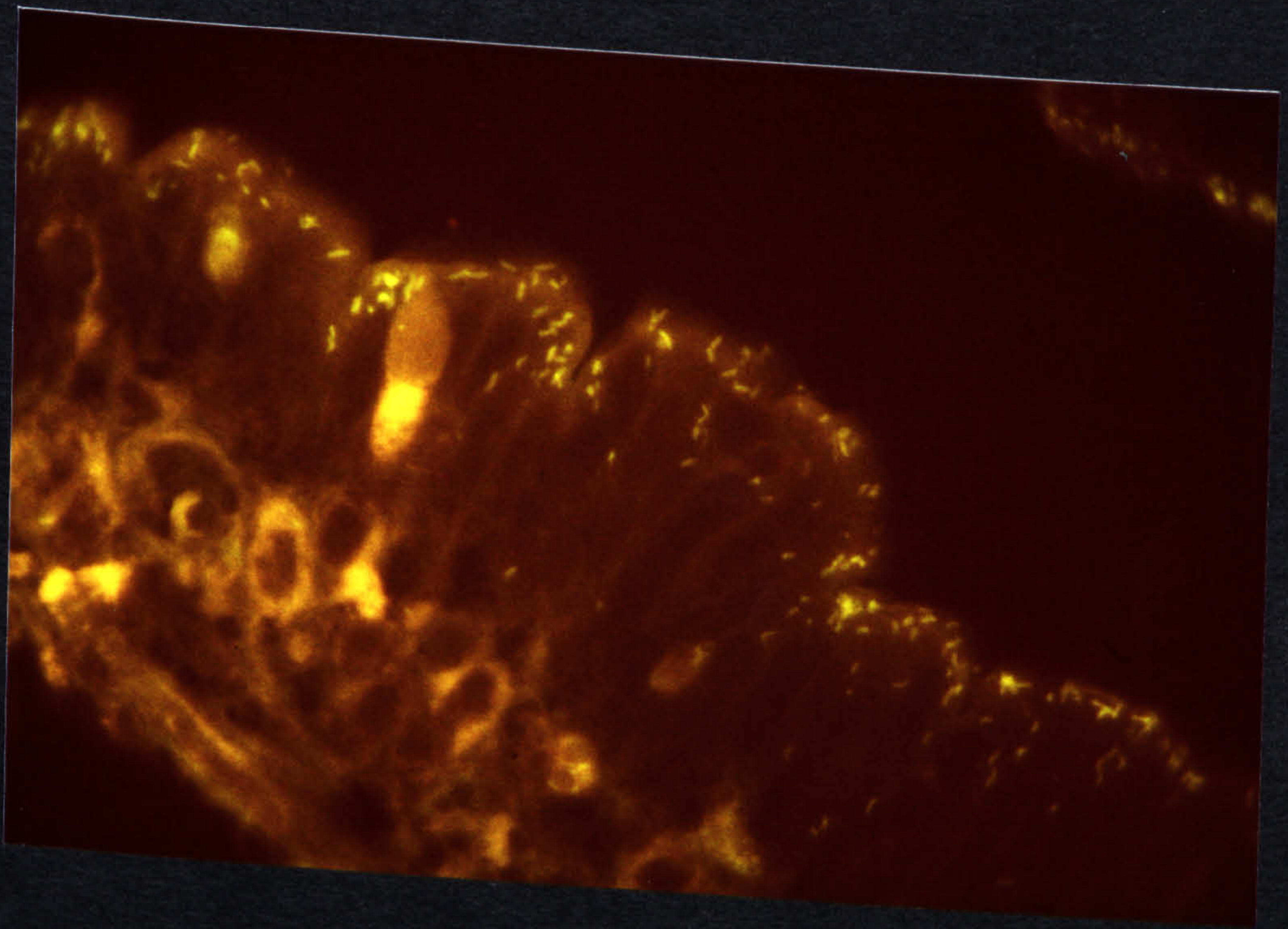


FIGURE 8.

Photomicrograph of unstained section of proximal colon from pig 1, treated in an indirect immunofluorescence assay.

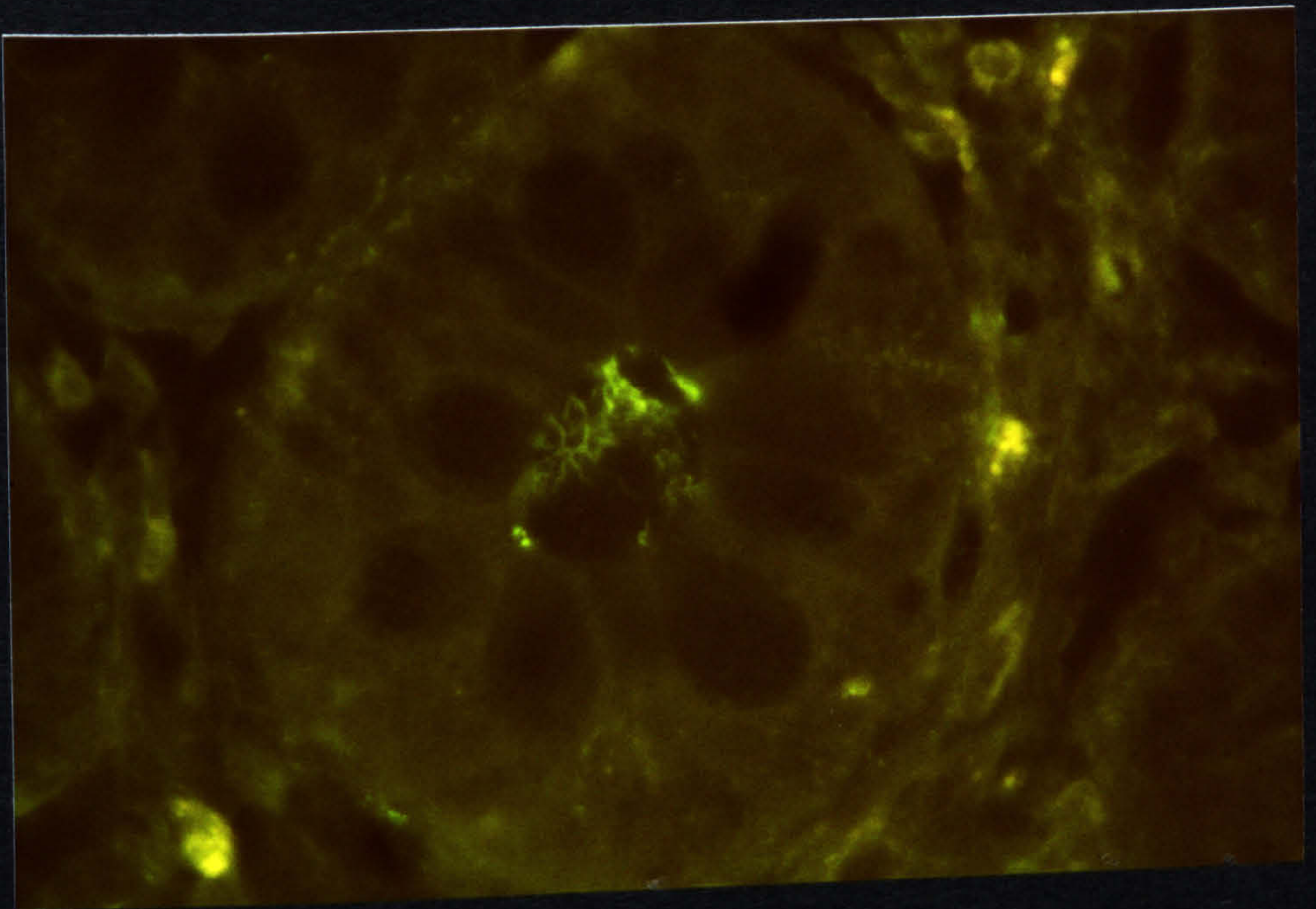
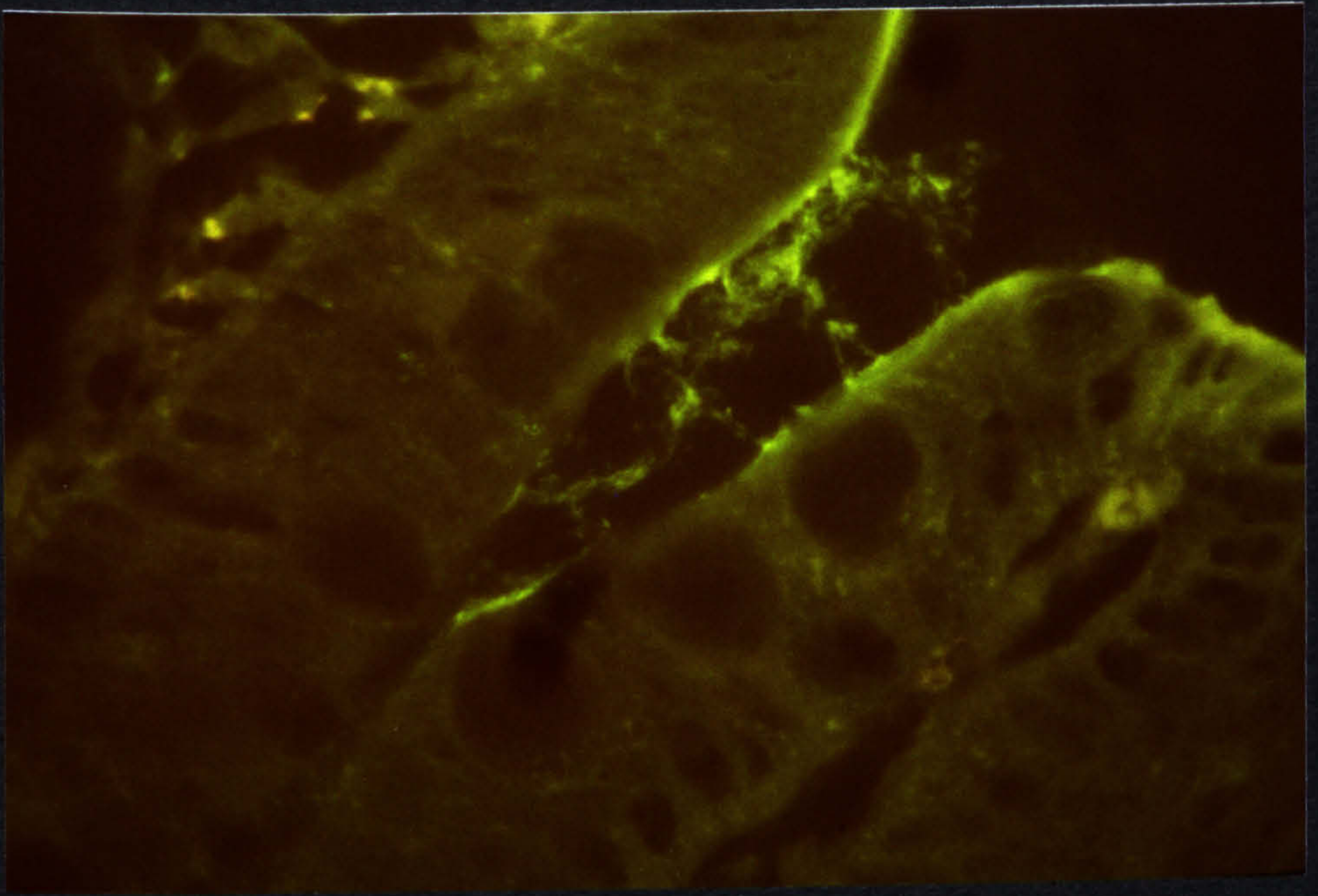
Rabbit antiserum to Campylobacter coli 9BF2 diluted 1:80 in phosphate-buffered saline, was the first antibody, and fluorescein-conjugated sheep anti-rabbit immunoglobulins the second antibody. Brightly fluorescent curved bacilli evident within the bowel lumen, adjacent to enterocytes.

x 600.

FIGURE 9.

Photomicrograph of unstained section of proximal colon of pig 8, treated in an indirect immunofluorescence assay, as above, with rabbit antiserum to Campylobacter hyointestinalis 9AL3, diluted 1:80 in phosphate-buffered saline as the first antibody. Brightly fluorescing curved bacilli evident within a crypt lumen.

x 600.



bacilli had colonised the ileal and large intestinal crypts. Attachment and entry of Campylobacter-like organisms into crypt enterocytes was also evident, with some proliferation of both bacteria within cells and of the enterocytes themselves. Morphologically, the curved bacilli within the lumina of the crypts of affected glands and the bacteria within the cells both appeared similar to the published structure of Campylobacter sp (Rowland and Lawson, 1986). Twenty days after infection there was similar intracellular colonisation of bacteria and proliferative activity, although no luminal bacteria were evident. A moderate sub-acute inflammatory reaction was evident throughout. Similar findings occur in the pathogenesis of proliferative enteritis of the hamster (Jacoby, 1978; Johnson and Jacoby, 1978), except that inflammation is not a feature of the early stages of the hamster lesion. The "additional" inflammation in the pigs may be due partly to their response to other bacterial antigens beside those of the infectious Campylobacter-like organisms, which were introduced with the inocula.

Culture of the successful mucosal inoculum only revealed a moderate number of C.coli, however an apparently greater number of Campylobacter-like organisms was evident in smears of this inoculum. This is a regular finding in mucosa affected by proliferative haemorrhagic enteropathy in which viable counts of Campylobacters rarely match the numbers of intracellular organisms (Love et al, 1977; Lawson et al, 1979). Colonisation of two of the affected piglets (Nos. 1 and 3) and two of the unaffected piglets by C.hyointestinalis indicates that this organism may establish quickly in the porcine intestine, despite being undetectable by the culture techniques used for the oral inocula. The most likely explanation is that C.hyointestinalis was present in very low numbers in the inoculum

Although it is possible that antibiotic-containing selective culture media may not recover all organisms that are viable in vivo. A previous experiment that failed to produce lesions noted the ready colonisation of the porcine intestine by C.hyointestinalis (Boosinger et al, 1985).

Following the completion of this study, a series of experiments reproducing proliferative enteritis in conventional pigs was reported (Mapother et al, 1987a,b; Joens et al, 1987). Piglets were dosed once with either crude homogenate or filtrates of mucosa affected by proliferative haemorrhagic enteropathy or necrotic enteritis. Concurrent corticosteroid treatment was given. Severe lesions of proliferative enteritis were noted 21 days after infection in pigs given crude homogenates, or 1.2µm filtrates. Pigs given 0.22µm filtrates developed a non-specific enteritis. C.hyointestinalis and C.coli were cultured from the intestines of affected piglets. Fresh mucosa was found to be of higher infectivity than frozen mucosa. It is possible that part of the different infectivity of mucosae in this study may have been due to the adverse effects of freezing being more pronounced in mucosae 716/86 and 761/86.

The results of cultures of alimentary tracts at necropsy and of faeces do not strengthen the association between any of the known Campylobacter sp and the disease. C.mucosalis did not persist in any pig other than briefly in the oral cavity, C.hyointestinalis was not cultured from the lesions or intestine of a pig with lesions (No. 2), and C.coli was not recovered from the small intestine of any pig with lesions and seemed only to colonise the lower bowel. Furthermore, antisera to two strains of C.hyointestinalis and C.coli which had been isolated from pigs 1 and 2, either from the faeces or from the lesions, did not identify the intracellular Campylobacter-like organisms. These

strains were chosen because if they had in fact turned out to be the intracellular organism, then the time and location of their isolation had optimised the chance of them being within the cells and/or possessing antigens associated with the intracellular location.

Recovery of C.hyointestinalis and C.coli followed a broadly similar pattern in the faeces and alimentary tracts of non-affected piglets to that observed in those with lesions.

That immunofluorescence studies also failed to associate cultured Campylobacter spp to organisms within enterocytes in experimentally affected pigs, is similar to previous immunofluorescence studies of the field disease (Lawson et al, 1985). Use of guinea-pigs as a second source of antisera, did not provide enhanced staining, but these animals are relatively free of C.jejuni infection (Fox, 1982), and naturally occurring proliferative enteritis is probably rare. Therefore the immunofluorescence reactions observed are likely to be more specific, with fewer of the cross-reactions and "natural" antibody reactions reported for antiserum produced in rabbits (Lawson et al, 1985). Comparison of the silver stain and immunofluorescence results for pig 1 indicates that many more curved bacilli were present in crypts adjacent to enterocytes containing bacteria than could be identified by in situ immunofluorescence.

Therefore, while this experiment clearly indicated that intracellular entry of Campylobacter-like organisms is closely associated with the early stages of proliferative enteritis, it is possible that the organisms are an as yet uncultured Campylobacter sp. However the close association of C.hyointestinalis or C.mucosalis to the field disease and C.mucosalis in previous experimental studies (Roberts et al, 1977; Lomax et al, 1982b), cannot as yet be explained. It is possible that a particular strain or modified form of a known



Campylobacter sp may be involved.

The failure to detect other agents, such as viruses or chlamydia ultrastructurally, and the failure of the successful inoculum to remain fully infective following 0.65 μ m filtration suggests that the bacteria observed were the principal agents involved.

The variable infectivity of the four inocula may have been due to reduced numbers of organisms in inocula filtered through 0.65 μ m pores. There may also be a difference in the infectivity of agents present within proliferative lesions of different types and at different stages of development.

This experiment indicated that gnotobiotic piglets can be safely exposed to filtered porcine intestine. Only one piglet became ill (No. 7) with lesions indicative of starvation/anorexia, probably secondary to a non-specific bacterial infection.

The demonstration of significant lesions at 10 days after infection suggests that gnotobiotic piglets can be used to study the crucial events of crypt colonisation, bacterial entry into cells and initiation of enterocyte proliferation occurring about this time. Culture and immunofluorescence tests applied at this time therefore have particular etiological significance; our results were not conclusive but suggest the presence of a further unidentified Campylobacter as the principal agent.

Further analysis of the lesions of pigs 1 and 2 is given in Chapter 7.

Given the failure to identify one or more Campylobacter sp as being directly linked with the onset of the disease in this experiment, further experimental inoculations with pure cultures were not considered useful. The successful inoculum contained numerous Campylobacter-like organisms of intracellular origin, but only few

C.coli were cultured. If culturable Campylobacter sp could be completely removed from this or other similar inocula, then this may constitute a "pure" infectious inoculum. However the technical difficulties in achieving this, for example by absorption with relevant antisera, seemed too formidable to overcome without the probable diminution of the inoculum's pathogenicity. Even so, such work may have to be undertaken for further understanding of the disease's pathogenesis.

CHAPTER 3

ORAL INOCULATION OF WEANLING HAMSTERS WITH
CAMPYLOBACTER SP AND/OR TISSUE FROM
PIGS AFFECTED BY PROLIFERATIVE ENTEROPATHY

Introduction

Proliferative enteritis presents as a similar clinical and pathological syndrome in hamsters and in pigs (Jacoby and Johnson, 1981). Affected weanling hamsters (3 to 10 weeks old) show hyperplasia of immature enterocytes in the ileum, with associated intracellular Campylobacter-like organisms. Naturally occurring disease in hamsters has only been reported from North America. Numerous experimental transmission studies have successfully reproduced the disease by oral inoculation of weanling hamsters with crude or filtered (0.65 μ) ileal mucosa from affected hamsters (Frisk and Wagner, 1977; Jacoby, 1978). While Campylobacter jejuni has frequently been isolated from the intestines of affected hamsters, the disease has not been reproduced by oral inoculation of hamsters with this agent (Lentsch et al, 1982; Regina and Lonigro, 1982; Fox et al, 1986). C.jejuni occurs in the intestines of many healthy hamsters (Fox et al, 1981).

Oral inoculation of weanling hamsters with crude intestinal mucosa from pigs affected with proliferative enteritis apparently reproduced the disease in 50 per cent of dosed hamsters, in a preliminary trial report (Gebhart et al, 1985b).

The identity of the intracellular Campylobacter-like organisms in naturally or experimentally affected hamsters is not known. Some immunofluorescence studies have suggested that the intracellular organism is antigenically different to known Campylobacter spp, and that the organism in pigs and hamsters is antigenically related (Lawson et al, 1985).

The results of Chapter 2 revealed several Campylobacter sp isolates as being associated with the lesions of experimentally induced proliferative enteritis in pigs. These isolates were considered candidates for being the major intracellular Campylobacter-like

organism.

Inoculum 284/86 contained few culturable Campylobacter sp, yet numerous Campylobacter-like organisms were visible in smears, and it reproduced proliferative enteritis in gnotobiotic pigs. This suggested that the visible Campylobacter-like organisms may be a specific candidate organism not yet able to be grown in vitro.

The studies in this Chapter set out to examine the transmissibility of porcine proliferative enteritis material to hamsters, the infectivity of porcine Campylobacter spp for hamsters and the infectivity of a dilution of porcine proliferative enteritis material free of culturable Campylobacter sp.

Materials and Methods

Hamsters

Hamsters (Mesocricetus auratus) were obtained from a small closed colony free of C.jejuni, the progeny of which were used for experimental studies. The parent colony had been infected with this organism and C.jejuni 664/83 was isolated from this source. Hamsters were bred randomly and maintained in stable laboratory animal facilities. Hamsters normally take in solid feed at circa 10 days old, and are weaned from their parents at circa 21 days old (for review of hamster biology, see Battles, 1985). Their solid feed diet was either Mouse/Hamster diet, Special Diet Services Ltd, Witham, Essex or Mouse Chow, Ralston Purina, St. Louis, Missouri, U.S.A.

Both these diets are proprietary formulations containing a balanced, complete nutrition, with 5 per cent and 3 per cent fibre respectively. Their exact formulae were considered confidential by each producer. The hamsters were kept on wood shavings and given feed and water ad lib throughout. Faeces and intestinal tract were

regularly monitored for the presence of C.jejuni by culture on selective media (Skirrow, 1977). All hamsters were weaned and dosed at 3 weeks of age.

Campylobacter spp Inocula

Each inoculum of 0.5ml of broth culture was administered orally via a blunt-ended 16 gauge 3cm needle. Controls were not dosed.

The identity of each isolate was confirmed by biochemical and slide agglutination reactions typical of each Campylobacter sp (Hebert et al, 1982; Lawson et al, 1976, 1985; Gebhart et al, 1985a). Each isolate had been subcultured 5 to 10 times and stored in glycerinated serum at -70°C. For each experiment a culture was transferred to Brucella broth (Difco, U.K.), incubated at 37°C in an atmosphere of 3 per cent or 6 per cent oxygen, 10 per cent carbon dioxide, 70 per cent hydrogen with nitrogen for 48 hours and given to hamsters as described above. Viable counts of Campylobacter sp in each inoculum, were calculated by the method of Miles and Misra (1938), and were $\log_{10} 7.0 \text{ ml}^{-1}$ for C.jejuni 1268/84J and $\log_{10} 7.0 \text{ ml}^{-1}$ for C.coli 1268/84K. These isolates were from a porcine ileum, designated 1268/84, affected with histologically confirmed proliferative enteritis. C.jejuni 664/83 was originally isolated from the ileum of a healthy hamster. It was given at a dose of $\log_{10} 8.9 \text{ ml}^{-1}$. C.hyointestinalis 9AL2 and 9AL3, and C.coli 9AF3a were originally isolated from the intestine or faeces of pigs affected by experimentally induced proliferative enteritis as described in Chapter 2. These were given at doses (per ml) of $\log_{10} 9.2$, $\log_{10} 9.2$ and $\log_{10} 7.9$ respectively. A C.fetus-like strain was isolated in low numbers from mucosa 284/86, on one occasion only, during the course of cultural examinations of the mucosae, see Table 4. It was given at a

dose of $\log_{10} 7.4$ per ml.

Proliferative Enteritis Inocula

Intestinal mucosa affected by histologically confirmed proliferative enteritis was collected aseptically from four pigs, see Table 4. Samples of mucosa were stored at -70°C for either four years (193/81) or three months, prior to use. Upon thawing, mucosal samples were weighed, washed in phosphate-buffered saline, homogenised to a fluid consistency and 1 to 1.5g administered orally to each hamster as described above. A 1ml sample of each inoculum was diluted for viable counts and incubated on Skirrow's medium (Skirrow, 1977) and RNBGT medium (McCartney et al, 1984) at 37°C in an atmosphere of 3 per cent or 6 per cent oxygen, 10 per cent carbon dioxide and 70 per cent hydrogen with nitrogen. Campylobacter sp isolations were identified as described above. For tissues 193/81 and 284/86, separate groups of hamsters were concurrently given 0.5ml of a broth containing $\log_{10} 7.5$ to 8.5ml^{-1} of C.jejuni 664/83 prepared as described above.

A further separate group of hamsters was given one ml of a 1:10,000 dilution of a homogenate of tissue 284/86 in Dulbecco's modification of Eagle's medium (Gibco, U.K.). No Campylobacter sp isolates were identified in cultures of this inoculum, however 1 to 5 Campylobacter-like organisms per high power field were identified in smears stained by modified acid-fast method.

Controls were not dosed.

Necropsy Procedures

Pathology - The small intestine from jejunum to lower ileum and portions of caecum and colon were collected into buffered formalin at necropsy. Subsequently the fixed small intestine was opened and rolled onto a wooden stick, prior to embedding, enabling the entire intestine

TABLE 4: Characteristics of porcine proliferative enteritis mucosae given to hamsters

Inocula no. and diagnosis		Estimated number of <u>Campylobacter</u> sp cultured (numbers expressed as $\log_{10}\text{ml}^{-1}$)		<u>Campylobacter</u> -like organisms in smears ^a
193/81	PHE	<u>C.fetus</u>	2.5	+
		<u>C.coli</u>	5.5	
284/86	PHE	<u>C.fetus</u> -like	1.0	+++
		<u>C.coli</u>	5.4	
716/86	PHE	<u>C.mucosalis</u>	8.4	+++
		<u>C.coli</u>	6.4	
761/86	PIA	<u>C.mucosalis</u>	7.1	+++
		<u>C.coli</u>	8.3	

a + 1-5, ++ 5-10, +++ >10 Campylobacter-like organisms per high power field

PHE Proliferative haemorrhagic enteropathy

PIA Porcine intestinal adenomatosis

collected to be examined on one slide.

Five μm sections were prepared and stained by haematoxylin and eosin and a silver impregnation stain (Young, 1969). The ratio of crypt to villus height was estimated using a measured objective eyepiece. Ten crypt/villus units were measured per section, and a mean value calculated.

Bacteriology - For experimental groups 1 and 2 (see Table 5) the terminal 5cm of ileum from each hamster was opened, washed in saline and a sterile swab rubbed over the mucosa. Swabs were spread onto selective media and cultured as described above.

For remaining experimental groups a one centimetre portion of ileum from each hamster was removed aseptically, homogenised and diluted in known volumes of phosphate buffered saline. The identity and concentration of Campylobacter spp in these dilutions was determined as described above.

Indirect immunofluorescence - Unstained sections of formalin fixed intestine from each hamster were digested with trypsin and prepared for immunofluorescence as described by Lawson et al (1985). Primary rabbit antisera used were those prepared against intracellular Campylobacter-like organisms purified from lesions of proliferative enteritis in a pig 1269/76 (Lawson et al, 1985), and against the four Campylobacter sp strains isolated (C.hyointestinalis 9AL2, 9AL3, C.coli 9AF3a, 9BF2) during the porcine experiment as described in Chapter 2. A further rabbit antiserum was prepared against C.jejuni 664/83, by an identical method to that described in Chapter 2 for the Campylobacter sp strains mentioned. Antiserum 1269/76 was applied to sections from all hamsters; other antisera were applied to sections from hamsters dosed with homologous Campylobacter sp and from control hamsters. Fluorescein-conjugated anti-rabbit immunoglobulin (Nordic Laboratories,

TABLE 5: Features of hamsters dosed with Campylobacter spp

Experimental Group	Inoculum	No. of hamsters	Date(s) of a necropsy	Histological changes in intestines	Mean crypt to villus ratio	Intestinal culture
1	<u>C.jejuni</u> 1268/84J	4	2,9	NAD	4/4 0.26	NSI
2	<u>C.coli</u> 1268/84K	4	2	Crypt hyperplasia	1/4 0.45	NSI
			2,9	NAD	3/4 0.35	NSI
3	<u>C.jejuni</u> 664/83	14	5,8,12,18	Acute enteritis	14/14 0.35	<u>C.jejuni</u> 14/14
4b	<u>C.hyointestinalis</u> 9AL2	4	10	NAD	4/4 0.30	NSI
5b	<u>C.hyointestinalis</u> 9AL3	3	10	NAD	3/3 0.25	NSI
6b	<u>C.coli</u> 9AF3a	4	10	NAD	4/4 0.25	NSI
7b	<u>C.fetus</u> -like 284/86	3	10	NAD	3/3 0.20	NSI
8	Controls for 1 - 3	14	2,8,12,18	NAD	14/14 0.25	NSI
9b	Controls for 4 - 7	6	10	NAD	6/6 0.28	NSI

a Date of necropsy expressed in number of days after dosing. At least 2 hamsters from each group were necropsied on each date.

b These groups were fed Mouse Chow (Purina) all others fed Mouse-Hamster Diet.

NSI No significant bacteria isolated

NAD No abnormality detected

U.K.) was used as the second stage antibody. Sections from any hamsters that apparently developed proliferative enteritis were also treated separately with Campylobacter sp antisera.

Identical serum and section controls to those described for assays of porcine tissue in Chapter 2 were incorporated.

Results

No Campylobacter sp was detected in the faeces of any hamster prior to experimentation or in control hamsters at necropsy. All hamsters remained clinically healthy throughout.

One hamster in experimental group 12 had gross thickening of the mucosa of the ileum at 21 days post-infection. No other gross lesions were detected. Controls were normal histologically.

Campylobacter sp inocula

All hamsters dosed with C.jejuni 664/83, had varying degrees of localised acute intestinal inflammation. Mucosal infiltration by neutrophils, lymphocytes and macrophages, and crypt lumina containing numerous curved bacilli were consistent features (Fig 10); the latter being readily visible on silver stains. Also, fluorescing curved bacilli were evident in these crypt lumina in sections treated with antiserum raised against C.jejuni, see Figure 11. Sections treated with antiserum against Campylobacter-like organisms 1269/76 showed no fluorescence. C.jejuni was recovered from all hamsters dosed with this organism alone, in concentrations of $\log_{10} 7.5$ per centimetre ileum where measured.

One hamster dosed with C.coli 1268/84K had moderate crypt cell hyperplasia in its jejunum and ileum at necropsy 2 days post infection. However, its crypt/villus ratio was not markedly greater than that of other bacteria-dosed hamsters, see Table 5. All hamsters dosed with

FIGURE 10.

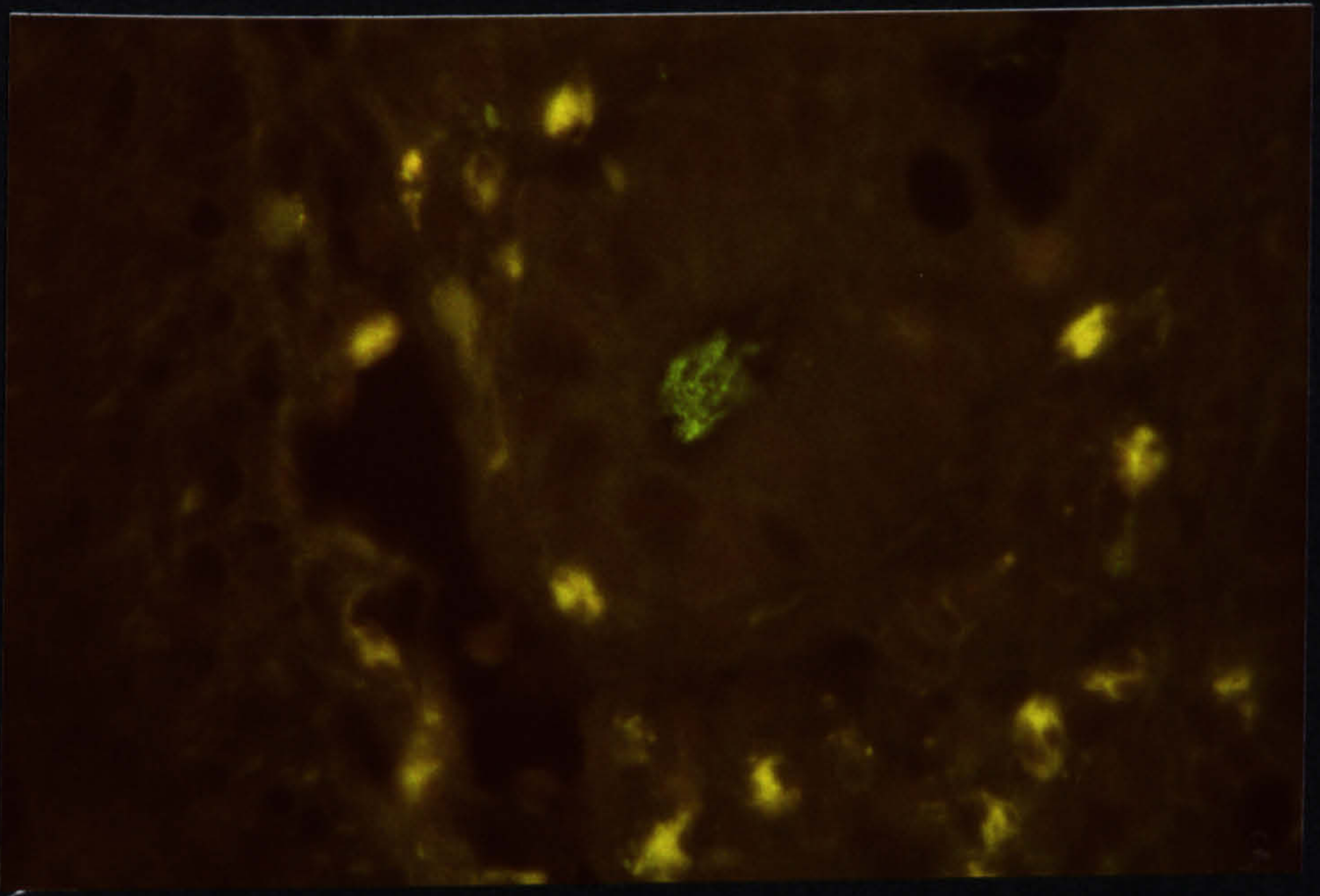
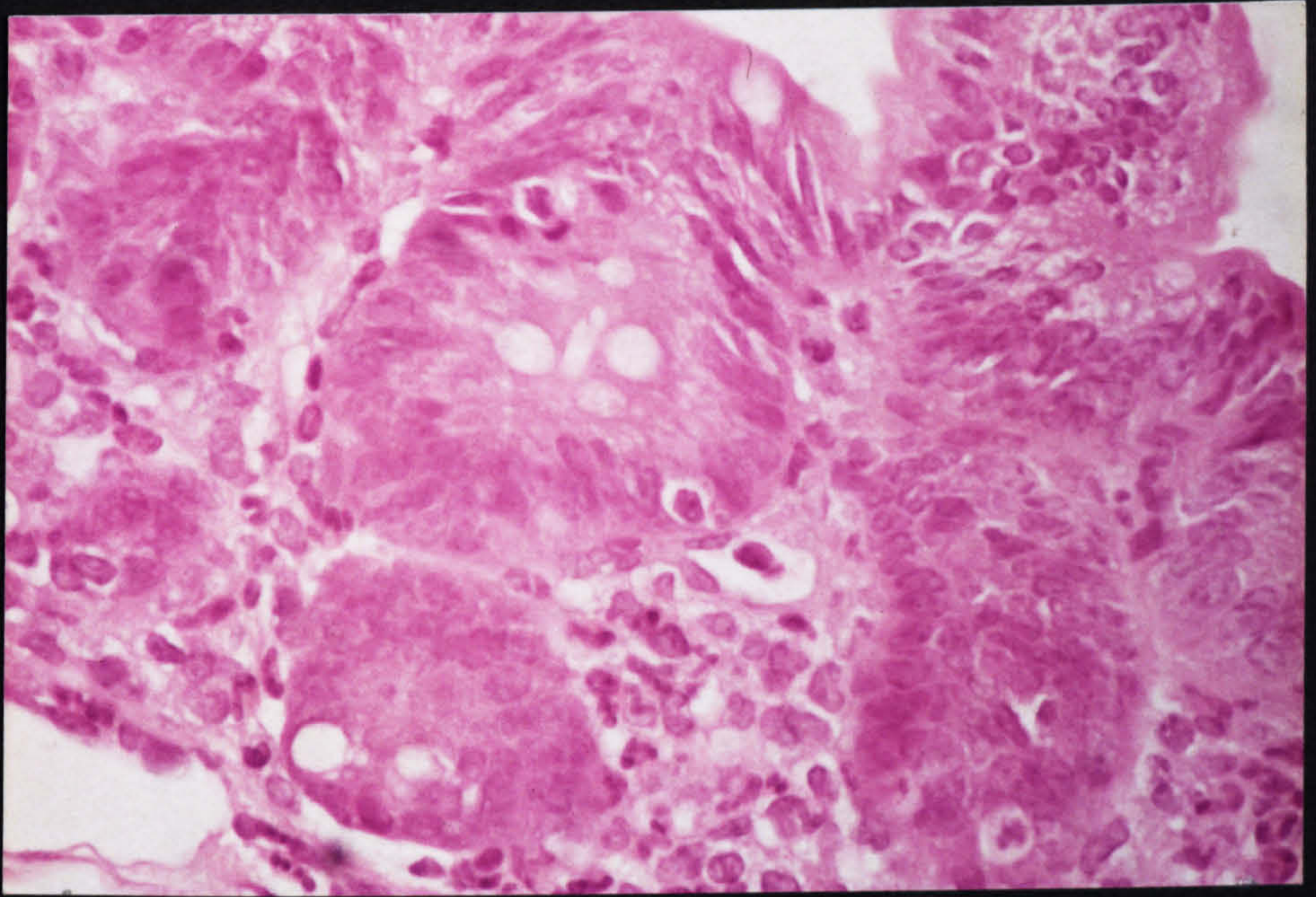
Photomicrograph of section of ileum from a hamster in group 3, dosed 18 days previously with C.jejuni 664/83, see Table 5. Moderate infiltration of mucosa by lymphocytes, macrophages and neutrophils.

Haematoxylin and eosin, x 400.

FIGURE 11.

Photomicrograph of unstained section of ileum from a hamster in group 11, dosed 23 days previously with mucosa 193/81 and C.jejuni 664/83, see Table 6. Section treated in an indirect immunofluorescence assay with rabbit antiserum to C.jejuni 664/83, diluted 1:80 in phosphate-buffered saline, as the first antibody, and fluorescein-conjugated sheep anti-rabbit, immunoglobulin as the second antibody. Brightly fluorescing curved bacilli within crypt lumen. No proliferation of enterocytes or intracellular bacteria evident.

x 600.



C.coli 1268/84K had crypt lumina containing numerous curved bacilli, and fluorescing curved bacilli were evident in these crypt lumina in sections treated with C.jejuni/coli antiserum.

No intracellular organisms were detected by silver stain or immunofluorescence assays with any antiserum, in any hamster in groups 1 to 9. Occasional hamsters in groups 4 to 8 and controls had intestinal crypt lumina containing numerous curved bacilli, evident in silver stains. No other fluorescence, isolations or lesions were evident, see Table 5.

Proliferative enteritis inocula

Lesions of proliferative enteritis were detected only in hamsters dosed with tissue 284/86, see Table 6. Lesions in the affected hamsters in groups 12 and 13 were confined to the ileum. Marked hyperplasia of crypt enterocytes, with numerous mitotic figures were evident (Fig 12). Numerous intra-cytoplasmic Campylobacter-like organisms were detected within enterocytes in affected portions of intestine by both silver stains and immunofluorescent staining with antiserum 1269/76, see Figure 13. Hamsters in group 12 that had lesions at 21 days post-infection also had mucosal infiltration by lymphocytes and macrophages.

These hamsters with developed lesions had few curved bacilli within local intestinal crypt lumina, and no fluorescence was evident in sections treated with C.jejuni, C.hyointestinalis or C.coli antiserum.

However, C.jejuni was recovered from all hamsters dosed with both C.jejuni and porcine tissue in concentrations of log₁₀6.0-7.0 per centimetre ileum. All such hamsters also developed mucosal changes similar to those described for hamsters dosed with C.jejuni alone.

TABLE 6: Features of hamsters dosed with porcine proliferative enteritis tissue + Campylobacter sp

Experimental Group	Inoculum ^a	No. of hamsters	Date(s) of necropsy ^b	Histological changes in intestine	Mean crypt to villus ratio	Intestinal culture
10 ^c	Tissue 193/81	5	23	NAD	0.32	NSI
11 ^c	Tissue 193/81 + <u>C.jejuni</u> 664/83	10	7,23	Acute enteritis	0.35	<u>C.jejuni</u> 14/14
12	Tissue 284/86	8	10,21	Proliferative enteritis	0.65	NSI
13	Tissue 284/86 + <u>C.jejuni</u> 664/83	8	10,21	NAD	0.45	<u>C.coli</u> 60/87 1/4
			10,21	Proliferative enteritis	0.85	<u>C.jejuni</u> 4/8
14	Dilute tissue 284/86	3	10,21	Acute enteritis	0.35	<u>C.jejuni</u> 4/8
15	Tissue 716/86	4	10,21	NAD	0.25	NSI
			10,21	Crypt hyperplasia	0.45	NSI
16	Tissue 761/86	5	10,21	NAD	0.30	NSI
17 ^c	Controls for 10 + 11	5	23	NAD	0.30	NSI
18	Controls for 12 - 16	11	10,21	NAD	0.28	NSI
					0.32	NSI

^a Characteristics of inocula given in Table 4

^b Date of necropsy expressed in number of days after dosing

^c These groups were fed Mouse-Hamster Diet, all others fed Mouse Chow (Purina)

NSI No significant bacteria isolated

NAD No abnormality detected

FIGURE 12.

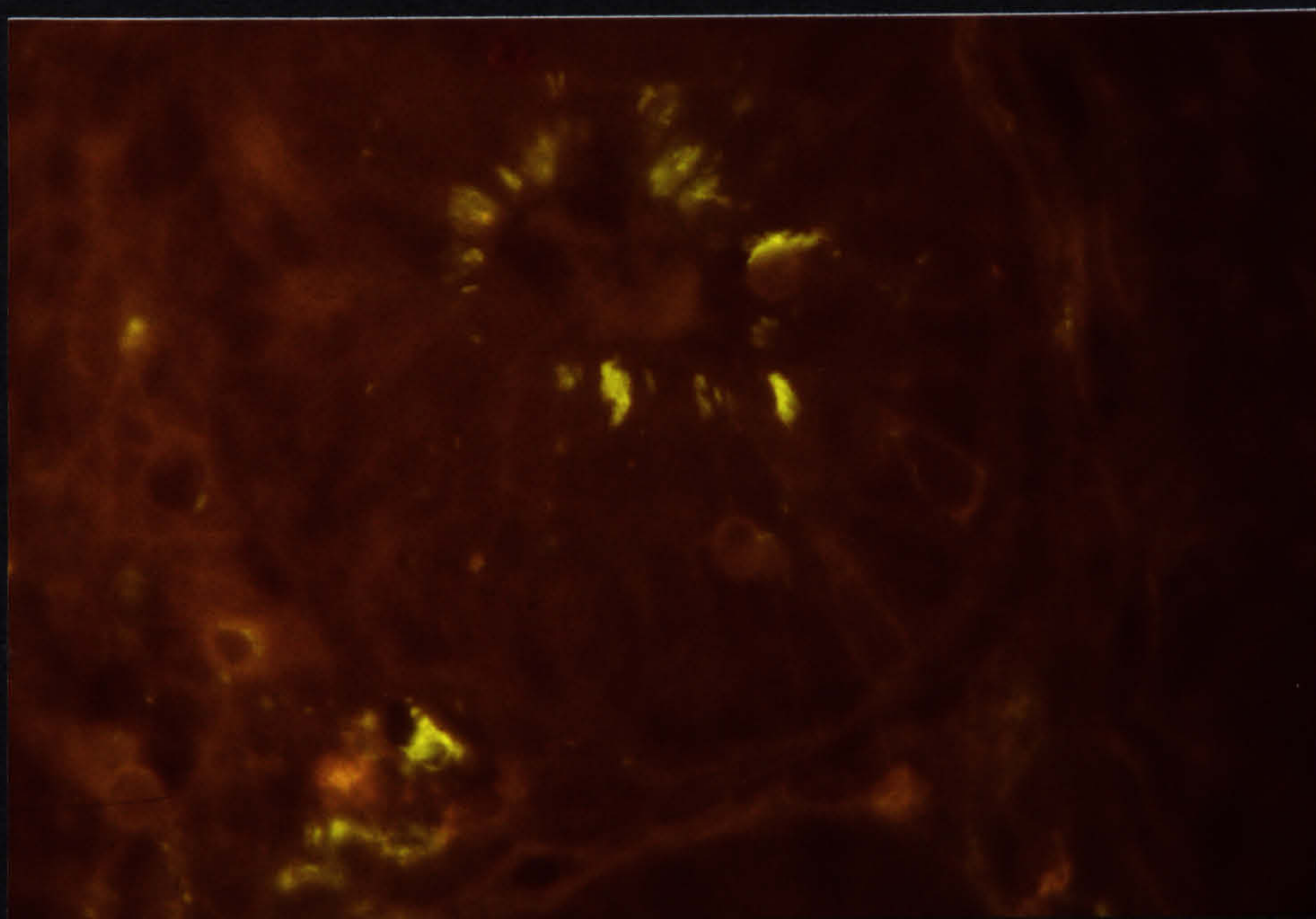
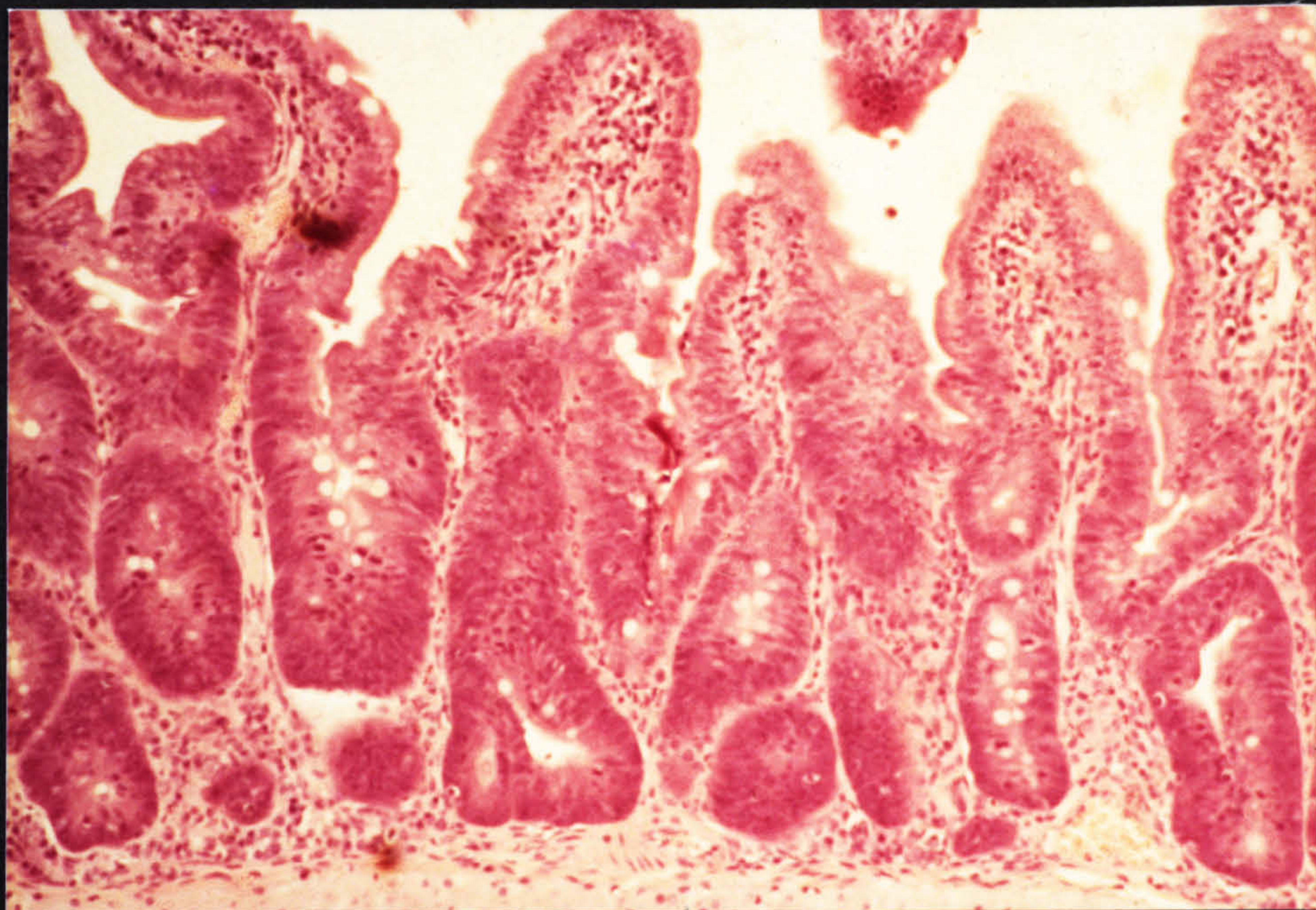
Photomicrograph of section of ileum from hamster in group 13, dosed 21 days previously with mucosa 284/86 and C.jejuni 664/83, see Table 6. Several crypts show lengthening and enlargement due to proliferation of enterocytes, with reduced numbers of goblet cells. Moderate mucosal infiltration by mononuclear cells and neutrophils also evident.

Haematoxylin and eosin, x 200.

FIGURE 13.

Photomicrograph of unstained section of ileum from hamster in group 13, dosed 21 days previously with mucosa 284/86 and C.jejuni 664/83, see Table 6. Section treated in an indirect immunofluorescence assay, with rabbit antiserum to Campylobacter-like organisms 1269/76, diluted 1:150 in phosphate-buffered saline, as the first antibody, and fluorescein-conjugated sheep anti-rabbit immunoglobulin as the second antibody. Brightly fluorescing curved bacilli evident within apical cytoplasm of proliferating crypt enterocytes. Fluorescing material evident within some associated mucosal mononuclear cells.

x 600.



Fluorescing curved bacilli were also evident in crypt lumina of all hamsters (other than those with proliferative enteritis) dosed with C.jejuni and porcine tissue, in sections treated with C.jejuni antiserum. Spiral and curved bacilli were also detected in intestinal crypt lumina in silver-stained sections in 15 control hamsters, these organisms did not react with C.jejuni antisera in immunofluorescence assays.

One hamster in group 12 which did not have significant lesions, 21 days post-infection, had C.coli 60/87 cultured from its ileum at a concentration of $\log_{10} 2.0$ per centimetre ileum.

Hamsters dosed with other mucosal tissue (groups 10, 15 and 16) did not develop lesions, except for one hamster dosed with tissue 716/86 which developed moderate crypt cell hyperplasia in its ileum, with a moderate lymphocyte and neutrophil infiltration into the ileal mucosa 21 days post-infection. All hamsters dosed with this tissue had crypt lumina containing numerous curved bacilli. No intracellular organisms were detected by silver stain or immunofluorescence assays with any antisera in any hamsters in groups 10, 11 and 14 to 18.

No other isolations or lesions were evident, see Table 6.

Discussion

Weanling hamsters proved to be susceptible to the agent of porcine proliferative enteritis by cross-species transmission. Several factors appeared to enhance this susceptibility; animals on the Purina diet and receiving a concentrated inoculum of tissue 284/86 showed the highest incidence of the disease. The disease which appeared in dosed hamsters was nearly identical to the naturally occurring one in pigs (Rowland and Lawson, 1986) and hamsters (Jacoby and Johnson, 1981). Oral dosing of certain Campylobacter sp cultured from pigs or hamsters did not appear

to be capable of reproducing proliferative enteritis.

The variation of the infectivity of the various porcine tissue inocula may partly be due to a dietary effect. Although the Purina diet was not used throughout, it appeared to enhance infectivity, a phenomenon similar to previous findings in groups in both hamster proliferative enteritis and murine colonic hyperplasia transmission experiments conducted on various diets (Barthold et al, 1977; Jacoby and Johnson, 1981). The successful diets may damage crypt cells or enhance their ability to take up antigens, provide substrates for growth of Campylobacter or alter local enzymes or redox potentials. Also pig tissue 284/86 was diluted prior to use in group 14 and tissue 193/81 had fewer Campylobacter-like organisms evident, suggesting that for reproduction of proliferative enteritis, a dose containing numerous intracellular Campylobacter-like organisms is required. Similar conclusions were made from hamster tissue transmission studies (Frisk and Wagner, 1977; Jacoby, 1978).

The identity of the intracellular organisms remains unresolved from these data. No hamster has developed proliferative enteritis following inoculation of pure cultures of Campylobacter spp, in this, or previous studies (Lentsch et al, 1982; Regina and Lonigro, 1982; Fox et al, 1986). The failure to isolate C.mucosalis or C.hyointestinalis from any hamster suggests that these organisms are not associated with proliferative enteritis in this species. The inoculations of C.jejuni of hamster origin or C.coli of pig origin consistently produced some crypt cell hyperplasia and acute enteritis, but this may have been a reaction unrelated to proliferative enteritis, as no intracellular bacteria were observed. The reproduction of proliferative enteritis in hamsters apparently free of C.jejuni indicates that this organism is also not involved in the initiation of the disease in this species.

Furthermore, the failure of hamsters in group 11 to develop proliferative enteritis suggests that no relationship exists between C.jejuni and an unidentified factor in proliferative mucosa e.g. a virus, in the aetiology of proliferative enteritis.

The exact relationship of the spiral bacteria within crypt lumina and those within adjacent cells in experimentally reproduced proliferative enteritis was not clear. C.jejuni could only be visualized, by immunofluorescence in crypt lumina and recovered in culture, in hamsters dosed with C.jejuni. Immunofluorescence staining with antisera to C.jejuni suggested that the intracellular bacteria were antigenically different to C.jejuni; however C.jejuni was recovered in significant numbers from some affected ilea. Hamsters with developed lesions of proliferative enteritis did not have significant numbers of Campylobacter-like organisms in crypt lumina. In those hamsters dosed with porcine tissue alone, no organisms could be identified within crypt lumina, with any antisera. These results suggest that the intracellular organisms are distinct from C.jejuni. However, other populations of Campylobacter organisms were also possibly involved, particularly the intracellular Campylobacter-like organisms in the original inocula.

While it is likely that some C.jejuni/coli strains can colonise the mucus of intestinal crypts and are difficult to distinguish morphologically from spiral bacteria present in the crypts of healthy animals (Lee et al, 1986) the cultural and immunofluorescence results suggest that C.jejuni can form significant colonies in ileal crypts. Acute inflammation has been reported in previous experimental studies of C.jejuni infection of the hamster (Regina and Lonigro, 1982). The failure of any of the pig-derived Campylobacter sp tested, to colonise the hamster ileum, in contrast the hamster-derived C.jejuni 664/83

reduces the possibility that any of the former are the intracellular organism.

It is possible that only a particular strain of one or more Campylobacter sp is capable of crypt cell entry and subsequent proliferation and that this property is readily lost in vitro. Citrobacter freundii 1140 was the only strain of 23 tested of this organism capable of causing murine colonic hyperplasia (Barthold et al, 1977). The failure of hamsters in groups 15 and 16 to develop proliferative enteritis also suggests that, as discussed in Chapter 2, there may be a difference in the infectivity of agents present within proliferative lesions of different types and at different stages of development.

The crypt cells of weanling hamsters and pigs appear to be naturally susceptible to a common agent, and entry of the crypt cells by Campylobacter sp is an essential stage that may not always take place despite colonisation of the crypt lumina. An alternative explanation is that the causative agent is an obligate intracellular parasite whose presence is obscured by the numerous Campylobacters that can be recovered from the alimentary tract.

CHAPTER 4

ANTIGENIC ANALYSIS OF CAMPYLOBACTER SPP AND CAMPYLOBACTER-
LIKE ORGANISMS ASSOCIATED WITH PROLIFERATIVE ENTEROPATHY:
IMMUNOFLUORESCENCE AND ENZYME IMMUNOASSAYS

Introduction

The failure of experimentation with pig and hamster models to positively identify the Campylobacter-like organisms present in the lesions of proliferative enteritis (see Chapters 2 and 3) suggested that analysis of these organisms by immunological techniques may be a fruitful procedure. If antigens of the intracellular organisms could be identified in cultivated Campylobacter sp, a relationship may be established.

Previously, hyper-immune rabbit antisera have been raised against various Campylobacter sp and Campylobacter-like organisms associated with proliferative enteritis and reacted in agglutination and immunofluorescence assays (Lawson et al, 1976, 1985; Chang et al, 1984b). Most rabbit antisera to C.mucosalis or C.hyointestinalis did not react with other Campylobacter sp or with Campylobacter-like organisms purified from lesions; similarly antisera to the latter organisms did not react with cultured Campylobacter spp in one study (Lawson et al, 1985). That study also found that some rabbit antisera prepared against C.mucosalis or various unrelated bacteria reacted with the Campylobacter-like organisms in a non-specific manner. In general however, immunization of rabbits with whole cells of Campylobacters produced specific antibodies to the relevant immunogen. Therefore for the Campylobacter-like organisms to be identical with one or other Campylobacter sp, yet have apparently different antigenic coats, some in vivo or in vitro alteration of surface antigens would have to be considered.

Investigation was required of rabbit antisera reactions with whole cells of Campylobacter spp, and of possible causes of changes in surface antigens. One such cause is growth of bacteria in an environment of restricted iron availability (Hantke, 1985). Therefore

growth of "iron-deprived" Campylobacter spp was investigated with regard to changes in antigen recognition.

Materials and Methods

Bacterial strains - The Campylobacter spp strains used in this study had been isolated from porcine and hamster material at the Royal (Dick) School of Veterinary Studies, Edinburgh and in one case, elsewhere, see Table 7. These strains had been deposited in that School's culture collection, some had also been deposited in the National Collection of Type Cultures, Colindale, London. Cultures had been identified, passaged and stored as described in Chapters 2 and 3.

Iron-deprived bacterial strains - Selected strains of each Campylobacter sp (see Table 7) were deprived of iron by the cultural method of Field et al (1986). Briefly, deferrated ethylene-diamine diacetate (EDDA, Sigma Chemical Co., U.K.) was added to Brucella broth growth medium (Oxoid, U.K.) at a concentration such that free iron was chelated sufficiently to restrict bacterial growth. To ensure this effect, growth of each Campylobacter sp strain was monitored in supplemented broths and in unsupplemented controls, by measurement of each broth's optical density in a Nephelometer (M.S.E. Instruments, U.K.) over a 27 hour period. Only when growth was significantly retarded in broths containing EDDA, was each strain considered "iron-deprived". The concentration of EDDA which achieved this effect was either 0.0125mg (C.jejuni/coli strains) or 0.0302mg per millilitre of broth (C.hyointestinalis strains). C.mucosalis strains did not become "iron-deprived" with the method used, at any concentration of EDDA tested. Bacteria were harvested from broths by centrifugation (4,000g, 10 min.). Each bacterial pellet was suspended in phosphate-

TABLE 7: Campylobacter sp strains

Species	Designation	NCTC designation ^a	Tissue of Origin	Pathology at necropsy
<u>C.mucosalis</u>	1248/72 ^b	11000	porcine ileum	intestinal adenomatosis
	124/73B ⁴		porcine ileum	necrotic enteritis
<u>C.hyointestinalis</u>	124/73A ⁴	11562	porcine ileum	necrotic enteritis
	9AL2 ^b		porcine colon	proliferative enteritis
	9AL3 ^b		porcine colon	proliferative enteritis
	632/74 ^c		porcine ileum	intestinal adenomatosis
<u>C.jejuni</u>	1268/84J		porcine ileum	proliferative haemorrhagic enteropathy
	664/83 ^b		hamster ileum	normal
	9BF2 ^b		porcine faeces	proliferative enteritis
<u>C.coli</u>	9AF3a ^b		porcine faeces	proliferative enteritis
	573/77 ^c		porcine colon	normal
	60/87 ^b		hamster ileum	acute enteritis
	1268/84K ^c		procine ileum	proliferative haemorrhagic enteropathy
	12AF4 ^{b,c}		porcine faeces	proliferative enteritis
<u>C.pyloridis</u> -liked	1480/87 ^c		porcine stomach	normal

a National Collection of Type Cultures, Colindale, London.

b Iron-deprived antigen prepared.

c Preliminary immunofluorescence assay only performed on antigen preparations of these strains.

d This strain was obtained from Dr. D.M. Jones, University of Manchester.

Antisera was not prepared against the bottom four strains in the Table, or C.jejuni 1268/84J.

buffered saline (PBS) containing 0.3 per cent v/v formalin, and whole cell antigen prepared as described below.

Campylobacter-like organisms - Intracellular Campylobacter-like organisms were partly purified from porcine intestines affected by proliferative enteritis (see Table 8), by the homogenisation, trypsinisation and filtration method described in Appendix 5. Organisms prepared by this method were stored in PBS containing 0.3 per cent v/v formalin. Modified acid-fast stained preparations indicated bacterial counts of 10^7 to 10^8 organisms per millilitre, almost exclusively with Campylobacter morphology.

This purification procedure was also carried out with other samples of these mucosae, except that PBS alone was used as a diluent throughout. Samples of these filtrates were cultured for Campylobacter sp as previously described, with counts presented in Table 8.

Normal porcine intestine - A crude homogenate of intestinal mucosa from a normal pig 204/79, see Table 8, was the control antigen preparation.

Preparation of antisera - Adult New Zealand white rabbits were inoculated intravenously with bacterial suspensions of 10^8 cells per millilitre from 24h plate cultures of strains indicated in Table 7 and antisera prepared according to the method described in Chapter 2. A further rabbit had been inoculated intravenously with a similar suspension of Campylobacter-like organisms 1080/76, see Table 8, and antisera prepared according to the same schedule. Further rabbits had been inoculated intramuscularly with a suspension of Campylobacter-like organisms 1080/76 or 1269/76 emulsified in Freund's complete adjuvant, as previously described (Lawson et al, 1979). As a control, a further

TABLE 8: Porcine intestinal material^a

Number	Pathology	<u>Campylobacter</u> -like organisms in mucosal smears ^b	Estimated number of <u>Campylobacter</u> sp cultured ^c (numbers expressed as log ₁₀ ml ⁻¹)	
1080/76 ^d	PHE	+++	<u>C.mucosalis</u>	2.4
			<u>C.coli</u>	2.0
1269/76	PHE	+++	<u>C.coli</u>	1.5
284/86	PHE	+++	<u>C.coli</u>	4.5
761/86	PIA	+++	<u>C.mucosalis</u>	6.4
			<u>C.coli</u>	5.2
204/79	None	None	None	

a Mucosa collected aseptically and stored at -70°C.

b Smears stained by modified acid-fast, +++ > 10 Campylobacter-like organisms per high power field.

c Mucosae (except 204/79) filtered in phosphate-buffered saline 0.8µm filtrate cultured.

d Not used as antigen.

PHE Proliferative haemorrhagic enteropathy.

PIA Porcine intestinal adenomatosis.

rabbit had been inoculated intramuscularly with a suspension of 10^8 cells per millilitre from a strain of "Haemophilus somnus" 578/77, emulsified in Freund's complete adjuvant, as previously described (Lawson et al, 1985). Pre-immune sera were also examined. Antisera were not prepared to iron-deprived strains.

Antigen preparation - Campylobacter sp were harvested from 24 to 48 hour plate cultures, cultured as described previously. Each Campylobacter sp (standard and iron-deprived preparations), Campylobacter-like organism and porcine intestinal suspension was suspended in PBS containing 0.3 per cent v/v formalin to a constant density as measured by a Nephelometer (M.S.E. Instruments, U.K.) corresponding to Brown's opacity tube number 2. The protein concentration of these suspensions was 0.3mg/ml (+/- 0.05mg/ml), as determined by a commercial assay incorporating a modified Lowry copper reagent (B.C.A., Pierce Chemical Co., U.S.A.), see Appendix 5. This corresponded to a concentration of 10^8 to 10^9 colony forming units per millilitre for Campylobacter sp on culture, and 10^8 Campylobacter-like organisms per millilitre, on direct examination.

Indirect immunofluorescence assay - 0.010 ml of each antigen preparation was air-dried onto Teflon-coated glass slides (Flow Laboratories, Scotland), fixed in acetone for 15 seconds, dried again, then incubated with doubling dilutions (starting at 1:10) of each antiserum in PBS. After 30 minutes incubation at 37°C, slides were washed in PBS, and incubated with rhodamine- or fluorescein-conjugated sheep anti-rabbit serum (Wellcome, U.K.). After 30 minutes incubation at 37°C, slides were washed in PBS and examined under a fluorescence microscope. The fluorochrome conjugates alone did not react with any suspension. The final serum dilution which yielded brightly

fluorescing (+++) antigen was taken as the endpoint for each serum. Immunoglobulin concentrations in test antisera were not adjusted prior to use.

In the instances of certain antigens, designated in Table 7 as "preliminary assay only", dilutions of rabbit antiserum prepared against Campylobacter-like organisms 1269/76 or 1080/76 only were used in the assay described.

Absorption assays - Whole cells of C.mucosalis 1248/72, C.hyointestinalis 124/73 A4 and C.jejuni 664/83 were harvested from 6 plates per isolate into separate vials containing 1ml PBS. Duplicate batches were prepared and all batches bulked into one vial. Bacteria were disrupted by ultrasonic waves (8 kilo watts) in four 30 second periods from a sonicator (M.S.E. Instruments, U.K.). Each sonicated suspension was mixed with 1ml of rabbit antiserum 1269/76 diluted 1:20 in PBS, for one hour at 20°C.

Each suspension was centrifuged (4,000g, 10 min.), the supernatant fluid was collected and serially diluted, in parallel with unabsorbed serum. Each dilution was incubated with air-dried smears of antigen preparations of porcine intestinal material 1269/76 or 204/79, and processed for immunofluorescence as described above.

Enzyme-linked immunosorbent assay - Each antigen preparation was diluted 1:50 in sodium carbonate buffer, pH9.6 and used as the solid phase in micro-titre plates in an enzyme-linked immunosorbent assay (ELISA, Voller et al, 1980). Briefly, serial dilutions (starting at 1:20) of each antiserum in ELISA dilution buffer (see Appendix 1) were used as first antibody, with peroxidase conjugated goat anti-rabbit serum as the second. Orthophenylene diamine in phosphate-citrate buffer (pH5.0), with added hydrogen peroxide, was used as substrate

with 15 minutes development time. Optical densities were determined with a Titretex Scanner (Flow Laboratories, Scotland). The final serum dilution which yielded an optical density of > 0.200 was taken as the endpoint for each serum (Klipstein et al, 1985). Immunoglobulin concentrations in test antisera were not adjusted prior to use.

All assays were conducted in duplicate, at least twice, and dilution endpoints calculated. "Preliminary assay only" antigens were not tested.

Results

Assays with standard antigen preparations

Indirect immunofluorescence assay - Antisera to each Campylobacter sp strain gave reciprocal endpoint dilutions of 160 to 320, for each homologous antigen. Reactions to other strains of the same Campylobacter sp were 80 to 160. Reactions to strains of other Campylobacter sp, to Campylobacter-like organisms and to porcine intestinal material were all less than 10.

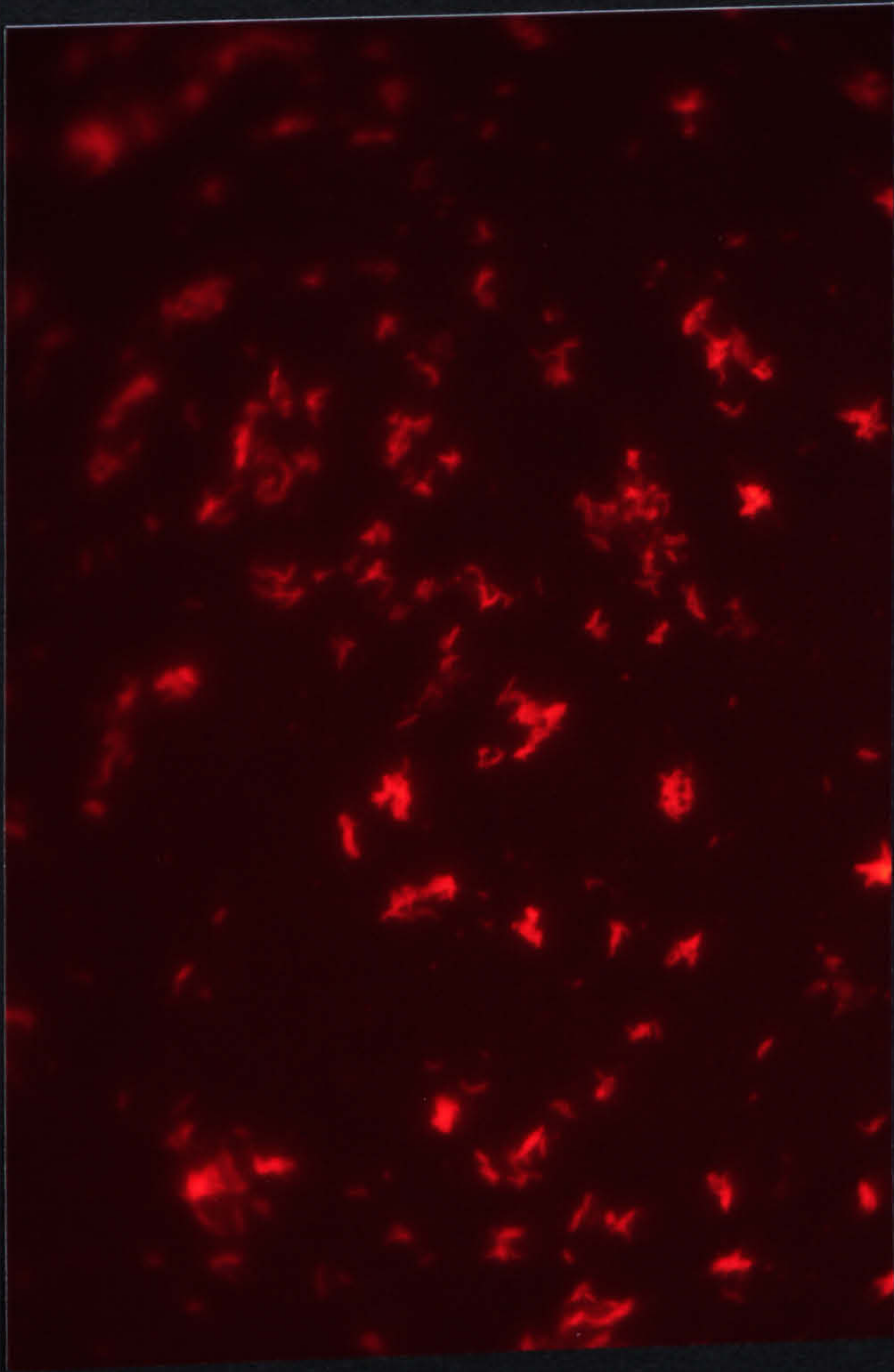
Antisera to Campylobacter-like organisms 1269/76 and 1080/76, gave endpoints of 640 to the antigen preparations 1269/76, 284/86 and 761/86 (Fig 14). Weakly fluorescing bacteria were observed in reactions of these antisera with standard preparations of C.hyointestinalis 124/73A4 and porcine C.pyloridis-like strain 1480/87, at dilutions up to 1:50. Reactions to mucosa 204/79, and all other standard Campylobacter sp antigens were less than 10. The control antiserum 578/77 did not react with any antigen.

There was a reaction in the pre-immune serum of one rabbit to the antigen preparation Campylobacter-like organism 1269/76 of 80. This reaction did not increase during the rabbit's immunization with C.jejuni 664/83. This serum was not used in any assay described above,

FIGURE 14.

Photomicrograph of smear of antigen preparation of Campylobacter-like organisms, partly purified from mucosa 1269/76 by homogenization, trypsinization and filtration, see Appendix 5. Indirect immunofluorescence assay with rabbit antiserum to Campylobacter-like organisms 1080/76 diluted 1:150 in phosphate-buffered saline, as the first antibody, and rhodamine-conjugated sheep anti-rabbit immunoglobulin as the second antibody. Brightly fluorescing curved bacilli, with some background material evident.

x 600.



as another antiserum was prepared. This second antiserum and all others used had pre-immune sera reactions to homologous antigen and to the antigen Campylobacter- like organism 1269/76 of less than 10.

Enzyme-linked immunosorbent assay - The results are given in Table 9.

Absorption assays - Incubation of antisera to Campylobacter-like organisms with Campylobacter sp antigen did not alter the reactivity of the antisera to the antigens tested, compared to unincubated controls (endpoint 640 for 1269/76).

Assays with iron-deprived Campylobacter sp

Campylobacter sp grown under iron deprivation conditions had numerous elongated, spiral forms evident in smears (Fig 15A). Immunofluorescence assays incorporating Campylobacter sp strains deprived of iron as antigen, had identical results to those reported above, for the antisera to Campylobacter sp and the control antiserum. However antisera to Campylobacter-like organisms 1269/76 or 1080/76 gave an endpoint of 40 for C.coli 9BF2 antigen deprived of iron (Fig 15B). Reactions of other iron-deprived strains with these antisera were less than 10. ELISA results for iron-deprived antigens consistently gave endpoints one or two dilutions less than those given in Table 9 for standard Campylobacter sp antigens.

Discussion

Rabbit antisera that were apparently specifically reacting with Campylobacter-like organisms in mucosae affected by proliferative enteritis did not react with the whole Campylobacter sp antigens tested. While the antigens tested in the immunofluorescence assay and ELISA were crude whole cell preparations, and several minor cross

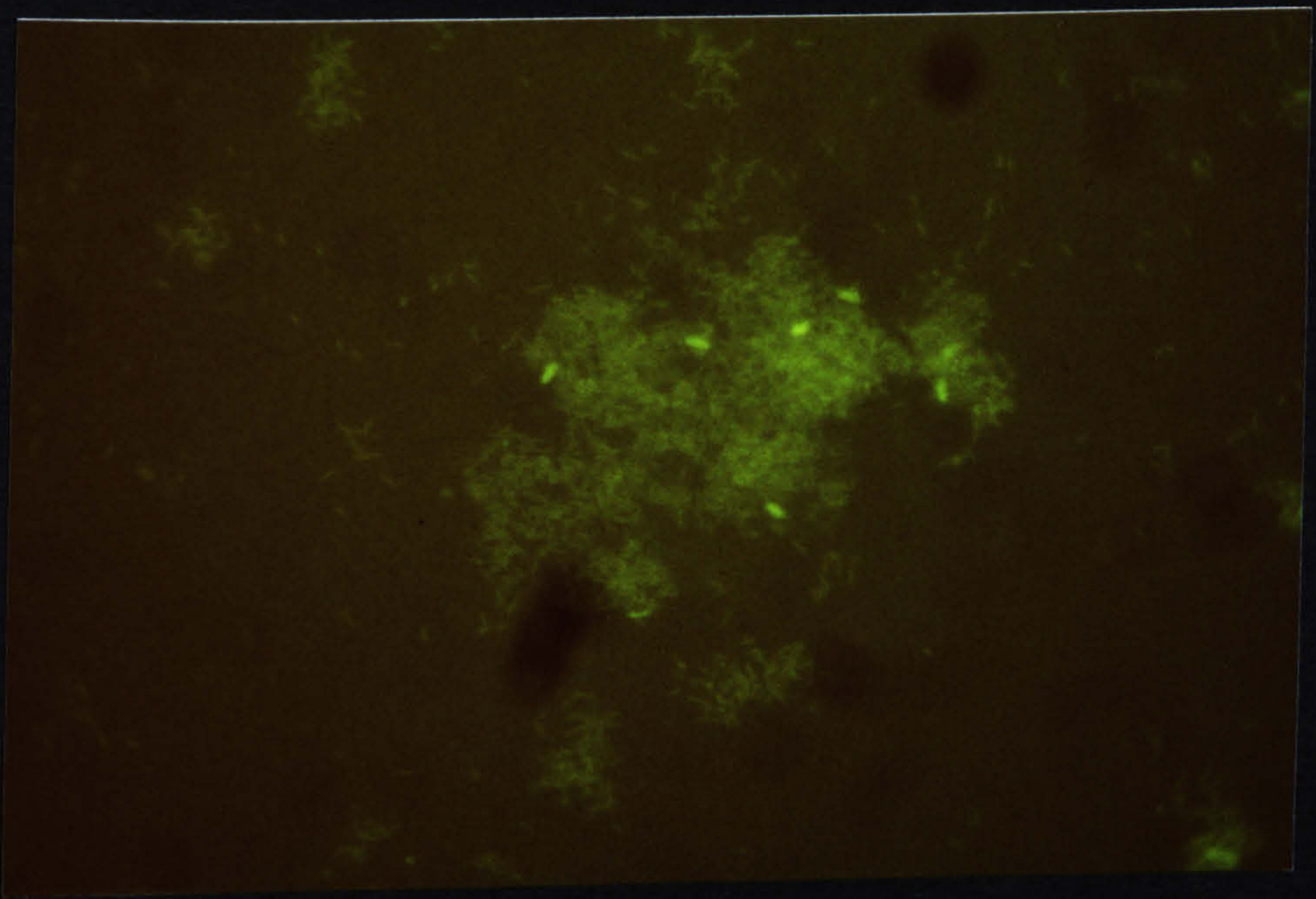
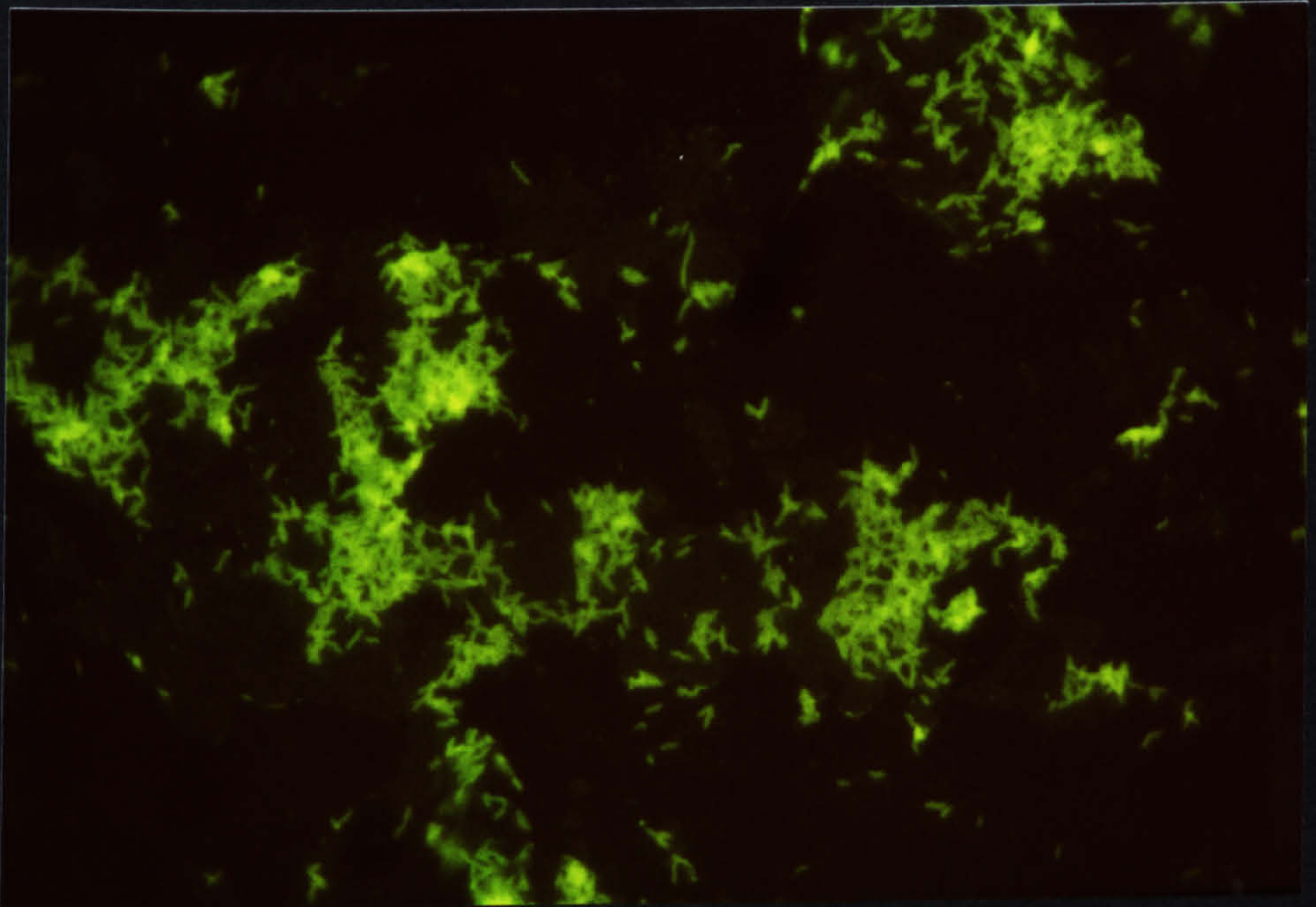
FIGURE 15.

A. Photomicrograph of smear of antigen preparation of Campylobacter coli 9BF2, that had been cultured in an iron-deprived medium. Indirect immunofluorescence assay, with rabbit antiserum to C.coli 9BF2 (rabbit immunized with C.coli grown on blood agar plates, i.e. not iron-deprived) diluted 1:80, as the first antibody, and fluorescein-conjugated sheep anti-rabbit immunoglobulin as the second antibody. Brightly fluorescing curved bacilli, with several elongated spiral forms evident.

X 600.

B. Photomicrograph of smear of the same antigen preparation as A, treated in an indirect immunofluorescence assay, with rabbit antiserum to Campylobacter-like organisms 1269/76 diluted 1:20 in phosphate-buffered saline as the first antibody. Moderately fluorescing curved bacilli evident.

x 600.



reactions were noted, the results generally suggested an antigenic difference between the intracellular organisms and Campylobacter sp tested.

This difference was also suggested by two other studies (Lawson et al, 1985; Gebhart, 1987). The use of rabbit antisera in these antigenic comparisons is complicated by the apparent presence in the serum of some normal rabbits of an immunological component that reacts with the intracellular Campylobacter-like organisms. One rabbit in this study and others in a previous study (Lawson et al, 1985) were clearly identified as having such a component in their pre-immune sera. This suggests that some rabbits have immunological memory, including antibody, to the intracellular or closely related organisms, possibly due to their previous natural infection with a Campylobacter. Many rabbits carry C.jejuni in their intestines (Fox, 1982) and a colitis associated with intracellular curved bacilli has been described (Moon et al, 1975). Given the wide host range of proliferative enteritis (Rowland and Lawson, 1986) a form of this disease may occur in rabbits.

Previous studies have raised rabbit antisera to various Campylobacter sp and used them to identify intracellular Campylobacter-like organisms (Rowland and Lawson, 1974; Chang et al, 1984a). These studies suggested that the intracellular organisms may be antigenically identical to one or other Campylobacter sp, however their results may have been confused by the presence of "natural" antibody in their rabbits.

The antigenic difference identified between the intracellular Campylobacter-like organisms and cultured Campylobacter spp may be due to some marked alteration in the bacterial cell membrane during their gut passage or on entry into the enterocytes. Depletion of iron in a bacterial environment can markedly alter their membranes (Hantke,

1985), and these alterations have been described for pathogenic Vibrio sp during enteric infections (Sciortino and Finkelstain, 1983), and for C.jejuni (Field et al, 1986). Therefore selected Campylobacter sp were depleted of iron to see if their membranes developed antigens recognised by antisera to the intracellular Campylobacter-like organisms. The spiral morphology that was induced, has been described previously (Field et al, 1986). The significance of the minor reaction in immunofluorescence assay of one iron-depleted C.coli strain to antiserum 1269/76 was not clear, and further tests were performed (see Chapter 5).

The absorption assays suggested that membrane antigens of C.mucosalis, C.hyointestinalis and C.jejuni are not involved in the reactions of antisera specific to intracellular Campylobacter-like organisms. This further supported the concept of the antigenic difference outlined above.

Interpretation of the ELISA results was limited by the use of crude whole cell antigens, which made standardization of the amount of antigen bound to each well difficult. Also the porcine intestinal preparations may have contained various types of bacteria and/or particular intestinal cell materials, which could have increased the likelihood of cross-reactions.

However, previous use of whole cell ELISA for C.jejuni (Walder and Forsgren, 1982), with a similar technique to that described here, found that it could reliably distinguish different Campylobacter sp. Therefore the observed difference in titres between cultured Campylobacter sp and the crude preparations of Campylobacter-like organisms may reflect a significant antigenic difference. Their results also noted that significant cross-reactions may occur between antisera to C.jejuni or C.coli and antigens of each of these species,

as is indicated for C.coli 9BF2 in Table 9. This suggests that strains of these two species may share antigenic sites.

CHAPTER 5

FURTHER ANTIGENIC ANALYSIS OF CAMPYLOBACTER SPP
AND PURIFIED CAMPYLOBACTER-LIKE ORGANISMS:
GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Introduction

Clarification of the identity of the intracellular Campylobacter-like organisms in lesions of proliferative enteropathy could be achieved by their purification and subsequent comparison to known Campylobacter spp.

Studies of the outer membranes of Campylobacter spp, particularly C.jejuni/coli, suggest that they consist of a major porin protein (44,000 MW), flagellin (60,000 MW) and other minor proteins between 20,000 and 90,000 MW as assessed by gel electrophoresis (Logan and Trust, 1982, 1983; Blaser et al, 1983); with other lipopolysaccharide components typical of Gram-negative bacteria (Logan and Trust, 1982; Perez et al, 1985). Reactions of these proteins and lipopolysaccharides with relevant immune sera has revealed that most of the major components are antigenic, with few cross-species reactions (Logan and Trust, 1983; Blaser et al, 1984). The flagellin protein is an exception as it has antigenic cross-reactivity between some Campylobacter sp, as well as with other spiral intestinal bacteria (Lee et al, 1987). Little previous study has been made of the outer membrane antigens of C.mucosalis or C.hyointestinalis. It is possible that the lack of reaction between the whole cells of the various Campylobacter spp, and antisera to the intracellular Campylobacter - like organisms (Chapter 4) is due to some significant antigens being internal components of the Campylobacter sp, and therefore "shielded" from possible reactivity.

Use of sensitive gel electrophoresis and immunoblotting techniques to analyse the antigens of the outer membranes and internal structures of the Campylobacter-like organisms in proliferative enteritis, would probably require that purer preparations than those used in Chapter 4 were available. Several biochemical separation techniques were thus

investigated. Following a preliminary trial which indicated that lectins would specifically react with some Campylobacter sp, lectin affinity chromatography was used to purify Campylobacter-like organisms, from mucosae affected by proliferative enteritis. Lectins have been shown previously to react with both certain strains of Campylobacter sp and with intestinal cell material (Wong et al, 1985; Etzler and Branstrator, 1974). Therefore parts of a mixture containing these components, could be expected to bind to the appropriate lectins held onto a column. The unbound fraction should be free of the bound components, compared to a control column without such lectins. Also, some of the bound components could be expected to be eluted with the appropriate competing sugar for the lectin binding site. Such an approach was explored here.

Materials and Methods

Lectin Affinity Chromatography

Selection of lectins

Eight whole cell antigen preparations of Campylobacter sp, Campylobacter-like organisms and porcine intestinal material (see Results, Table 11) were prepared to standard suspensions as described in Chapter 4. A suspension of an additional strain, C.coli 284/86F, isolated from mucosa 284/86, was also prepared from 48 hour sub-cultures, as lectin reactions can involve intra-species variations. One millilitre of each antigen suspension was washed twice in PBS supplemented with calcium and magnesium ions, and bovine serum albumin (Lectin buffer 1, see Appendix 1), then re-suspended in one millilitre of this buffer. Samples of each suspension (0.025ml) were mixed with 0.025ml of each of 11 lectin-fluorescein conjugates (see Table 10) in wells of microtitre plates. After incubation at 20°C for one hour, wet

TABLE 10: Lectins

Lectin ^a	Abbreviation	Origin	Sugar Specificity
Peanut agglutinin	PNA	<u>Arachis hypogaea</u>	D-galactose
<u>Bauhinia purpurea</u> agglutinin	BPA	<u>Bauhinia purpurea</u>	D-galactose N-acetyl D-galactosamine
Concanavalin A	ConA	<u>Canavalis ersiformis</u>	D-mannose D-glucose
Horse-grain agglutinin	DBA	<u>Dolichus biflorus</u>	N-acetyl d-galactosamine
Soybean agglutinin	SBA	<u>Glycine max</u>	N-acetyl D-galactosamine
<u>Griffonia simplicifolia</u> agglutinin I	GSI	<u>Griffonia simplicifolia</u>	D-galactose
<u>Griffonia simplicifolia</u> agglutinin II	GSII	<u>Griffonia simplicifolia</u>	N-acetyl D-glucosamine
Slug lectin	LPA	<u>Limus flavus</u>	Sialic acid
Osage orange agglutinin	MPA	<u>Maclura pomifera</u>	D-galactose
Wheat-germ agglutinin	WGA	<u>Triticum vulgaris</u>	N-acetyl D-glucosamine
Corse seed agglutinin I	UEAI	<u>Ulex europeus</u>	L-fucose

^a Supplied as fluorescein conjugates from Miles Laboratoris, U.S.A. Diluted for use, to a final concentration of 0.1mg ml⁻¹ in lectin buffer 2 (Appendix 1).

smears of each mixture were made on glass slides, and examined under a fluorescence microscope. A positive reaction was recorded if brightly fluorescent bacterial cells were evident.

Agarose columns

Initial sample preparation - Campylobacter-like organisms were partly purified from intestinal mucosae 284/86 and 761/86 only (see Table 8) by the homogenization and filtration method (see Appendix 5), except that PBS supplemented with calcium and magnesium ions (Lectin buffer 2), was used as diluent throughout. Standard suspensions (see Chapter 4) were prepared in this buffer, and applied to three sets of columns. Normal mucosa was not included.

Wheat-germ agglutinin - Wheat-germ agglutinin (WGA) insolubilized on cross-linked 4 per cent beaded agarose (Sigma Chemical Co., U.K.) was suspended in buffer 2 in a 5ml vertical glass column (Bio-Rad, U.K.). One millilitre of the WGA-agarose suspension (= 7.35mg lectin) was washed with 20ml of buffer 2, then one millilitre of each sample was applied individually to separate WGA columns. One millilitre of unbound fraction was collected at a flow rate of 0.2ml per minute. This unbound material was re-applied and collected four more times. Twenty millilitres of buffer 2 was then applied to the top of each WGA column and 20ml of unbound but washed material (washed fraction) collected; leaving only lectin-bound material on the columns. Five millilitres of 0.2M N-acetyl D-glucosamine (Sigma Chemical Co., U.K.) was then applied as the competing sugar to the top of each column and incubated with the gel for 4 hours at 20°C. Five millilitres of eluted fraction was then collected.

Agarose - Cross-linked 4 per cent beaded agarose (Sigma Chemical Co., U.K.) was prepared as a control column, and one millilitre of

sample 284/86 applied (as described above). The column was washed and eluted, and fractions collected as described above.

Griffonia simplicifolia II agglutinin - One millilitre of sample 284/86 was applied to a WGA-agarose column as described above, and one millilitre of unbound fraction collected. This was then applied to a column of Griffonia simplicifolia II agglutinin (GSII) insolubilized on cross-linked 4 per cent beaded agarose (Sigma Chemical Co., U.K.). One millilitre of GSII agarose gel (= 1.6mg lectin) had been suspended in a separate 5ml vertical glass column and washed in buffer 2 as described above. One millilitre of the GSII unbound fraction was collected at a flow rate of 0.2ml per minute, re-applied and collected four more times. Washing and elution of the GSII column was performed as described above, with buffer 2 and N-acetyl D-glucosamine respectively.

Analysis of samples and fractions - One millilitre of each initial sample preparation and the entire volume of each fraction were transferred to dialysis tubing (Spectra/Par, MW cutoff 14,000, Spectrum Medical Industries, U.S.A.) and dialyzed at 4°C for 24 hours with several changes of distilled water. These preparations were then frozen and water removed by vacuum freeze-drying (Virtis Co. Inc., U.S.A.) at -70°C, 15mTorr for 24 hours. Each preparation was then reconstituted in 0.1ml of distilled water.

Samples from each of these preparations were smeared onto glass slides and stained by modified acid-fast method. Further smears were stained by indirect immunofluorescence assay, incorporating rabbit antiserum to Campylobacter-like organisms 1269/76 (see Chapter 4). The protein concentration of each preparation was determined by a commercial assay incorporating a modified Lowry copper reagent (B.C.A., Pierce Chemical Co., U.S.A., see Appendix 5).

Samples of each preparation were cultured for Campylobacter sp, by

placing a drop of each preparation on blood agar and Skirrow's agar, and incubating them as described in Chapter 2.

Further samples of each preparation were diluted 1:50 in carbonate buffer, pH9.6 and used as solid phase in ELISA (Voller et al, 1980). Selected rabbit antisera (see Results, Table 12) were diluted serially in ELISA dilution buffer and used as first antibody in this assay, with subsequent ELISA steps performed as described in Chapter 4.

Each agarose column was set up and run at least twice.

Polyacrylamide Gel Electrophoresis

Antigen preparations

Sonicated antigens - Each whole cell antigen preparation of Campylobacter sp, Campylobacter-like organisms and porcine intestinal material described in Chapter 4 (Tables 7 and 8), individual samples and fractions from each lectin column prepared as described above, and a further antigen preparation of C.coli 9BF2 which had been prepared from an iron-deprived culture (see Chapter 4) was included in this study. Each preparation was washed twice in 25mM Tris-HCl buffer pH8.3, then disrupted by ultrasonic waves, in four 30 second bursts at 8 kilowatts amplitude (M.S.E. Instruments, U.K.). Prior to use in gel electrophoresis, each antigen was mixed 3:1 with a concentrated reducing sample buffer (see Appendix 1) and boiled for 4 minutes at 100°C. Samples were stored until required at -20°C.

Outer membrane proteins - Forty eight hour cultures of C.mucosalis 1248/72, 124/73B4, C.hyointestinalis 124/73A4 and C.jejuni 1268/84J were scraped from agar plates into 0.1M Tris-HCl buffer pH8.3 and outer membrane proteins (OMP) prepared from each as previously described (Mills and Bradbury, 1984). Briefly, bacterial cells were washed in, and each pellet re-suspended in 0.1M Tris-HCl buffer, pH8.3

(60ml). Thirty millilitres of 2M sucrose, 1.2ml of 1 per cent w/v sodium ethylene diaminetetra-acetate (sodium EDTA) and 4.8ml of 0.5 per cent w/v lysozyme (Sigma Chemical Co., U.K.) were added sequentially. The mixture was incubated for 45 minutes at 25°C then spheroplasts removed by centrifugation (20,000g, 20 min). The supernatant fluid was centrifuged (100,000g, one hour, 4°C) and the pellet suspended in 2ml sodium EDTA and dialyzed overnight against sodium EDTA. After dialysis, the preparation was centrifuged (100,000g, one hour, 4°C) and the pellet suspended in 0.1M Tris-HCl buffer, pH8.3. Samples for electrophoresis were prepared and stored as described above.

Flagellin - Forty eight hour cultures of C.mucosalis 1248/72 and C.hyointestinalis 124/73A4 were scraped from agar plates into distilled water and flagellin prepared from each as previously described (Logan and Trust, 1983). Briefly, cells were washed 5 times in water, frozen in water at -20°C for 3 days, then thawed and centrifuged (100,000g, one hour, 4°C). The pellet was suspended in water, centrifuged (10,000g, 10 min) and the supernatant fluid withdrawn and re-centrifuged (100,000g, one hour, 4°C). The pellet was incubated in 0.1N hydrochloric acid, for 18 hours at 4°C then centrifuged (100,000g, one hour, 4°C). The supernatant fluid was suspended in water pH7.0 for 30 minutes at 4°C, frozen and concentrated by freeze-drying. The purity of the flagella was assessed by electron microscopy of preparations stained by 0.2 per cent v/v phosphotungstic acid. Samples of flagellin for electrophoresis were prepared and stored as described.

Gel preparation and conditions

Vertical glass plates (160mm x 180mm) separated with 1.5mm spacers, were filled with a 20 per cent to 7 per cent (bottom to top) continuous gradient polyacrylamide gel, see Appendix 2 for formulae.

Immediately prior to pouring, 0.006ml of N,N,N,N-tetra- methylethylene diamine (TEMED) and 0.025ml of a 10 per cent w/v ammonium persulphate solution in water were added to each 20ml of gel, as polymerizing agents. After polymerization, a stacking gel, containing 0.025ml and 0.1ml of the respective polymerizing agents, was added with a comb for forming the sample wells. Each gel was placed on a vertical gel tank apparatus containing Tris-glycine buffer (see Appendix 1), and samples loaded. Five to 10 g of protein per sample, as indicated by the BCA test described previously, were used. Electrophoresis was performed at 20°C with a constant voltage of 200v for one hour, then 80v for 18 hours. Proteins of known molecular weight (Pharmacia, U.K.) were run on each gel. Gels were stained by a silver stain (Morrissey, 1981, see Appendix 3), and molecular weights determined for each antigen band from a standard curve.

Immunoblotting

Components separated by gel electrophoresis were transferred from unstained gels to nitrocellulose paper by semi-dry electroblotting on a graphite electrode apparatus (Ancos electroblotter, Dako Ltd., U.K.) according to the manufacturer's instructions. Briefly, two sheets of Whatman filter paper number 3 were soaked in transfer buffer (see Appendix 1) and placed on the anode. The nitrocellulose paper, the gel and two further sheets of soaked filter paper were placed on top, then the cathode assembled. This "sandwich" was electroblotted for 90 minutes at 20°C at a constant voltage of 24v.

Protein binding sites on the nitrocellulose paper not occupied by sample components were blocked by incubating papers for 4 hours with 25mM Tris- HCl buffer supplemented with gelatin and goat serum (Blocking buffer, see Appendix 1). Nitrocellulose papers were then

incubated at 20°C for 18 hours in rabbit antiserum to each Campylobacter sp and Campylobacter-like organism diluted 1:200 in blocking buffer. In some cases, the immunoglobulin in serum 1269/76 was concentrated fourfold by ammonium sulphate precipitation (see Appendix 5) prior to incubation with the nitrocellulose papers (i.e. a 1:50 dilution). After being washed in PBS, papers were incubated at 20°C for 2 hours in peroxidase-conjugated goat anti-rabbit immunoglobulin serum (Nordic Immunologicals, U.K.) diluted 1:2,000 in blocking buffer. After being washed, papers were placed in colour development solution (0.05 per cent w/v 4-chloro-naphthol in 50mM Tris-HCl buffer, pH7.5 with 0.001 per cent v/v hydrogen peroxide).

In some instances, following the first antiserum and washing steps, nitrocellulose papers were incubated in iodine 125-conjugated donkey anti-rabbit immunoglobulin serum (Amersham, U.K.) diluted 1:2,000 in blocking buffer. Radioactivity was measured as 2 to 10 Ci per incubation. After being washed, papers were dried and placed into an X-ray cassette with a clean X-ray film (Ilford, U.K.). After incubation at -70°C for one to 7 days, films were developed.

No significant differences were detected in immunoblotting results with either technique.

Gels incorporating each antigen and the various antisera combinations were run at least twice.

Results

Lectin Affinity Chromatography

Selection of lectins

The results of reactions between lectins and test antigens are given in Table 11. Wheat-germ agglutinin and Griffonia simplicifolia II agglutinin clearly reacted with C.coli and Campylobacter-like

TABLE 11: Reactions of lectins with antigen preparations

Antigen preparation	Fluorescein conjugated lectin				
	PNA ^a	ConA	GSI	GSII	WGA
<u>C.mucosalis</u> 1248/72	-	-	-	-	-
<u>C.hyointestinalis</u> 124/73A ⁴	-	+++	++	++	-
<u>C.jejuni</u> 1268/84J	-	+++	-	++	-
<u>C.coli</u> 9BF2	-	-	-	-	+++
284/84F	-	-	-	-	+++
<u>Campylobacter-like organisms</u>					
1269/76	+	-	-	+++	-
284/86	++	-	-	+++	+
761/86	+	-	-	++	-
Normal pig intestine 204/79	-	-	-	-	-

a Abbreviations explained in Table 10.

All other lectins, no fluorescence evident (-).

+ Occasional, weakly fluorescing bacteria.

++ Moderately fluorescing bacteria.

+++ Brightly fluorescing bacteria.

organisms respectively. On the basis of these reactions two strategies were used as indicated in the Materials and Methods . First, porcine intestinal material containing numerous Campylobacter-like organisms was passed down a column containing wheat-germ agglutinin, with the intention of removing C.coli and intestinal material from the unbound fraction. Second, material was then passed down a column containing Griffonia simplicifolia II agglutinin to capture the Campylobacter-like organisms, to be removed by elution with N-acetyl-D-glucosamine (see Table 10).

Agarose columns

The results of analysis of the various lectin and control agarose columns compared to initial samples are given in Table 12. Numerous brightly fluorescing Campylobacter-like organisms were observed in smears of the unbound fraction from the WGA columns, with much reduced background material compared to the initial sample of mucosa 284/86 as shown in Figure 16. Similar organisms were observed in smears of the unbound fraction from the agarose control columns, however significant amounts of background material were still present. Relatively few organisms were observed in fractions collected from the GSII column.

The ELISA results indicated that C.coli antigens were not present in the unbound fraction of the WGA columns. The unbound fraction of the agarose control columns did bind C.coli antisera at low titre.

Protein concentrations of fractions showed considerable variability between repeated runs. Initial samples contained 0.18 to 0.39mg/ml, unbound fractions 0.02 to 0.33mg/ml, washed fractions 0.007 to 0.37mg/ml and eluted fractions 0.08 to 0.32mg/ml.

Culture of all fractions from columns was negative for Campylobacter sp. Culture of initial samples is given in Table 8.

TABLE 12: Lectin affinity chromatography

Sample analysed	Immunofluorescence - rabbit antiserum to <u>Campylobacter</u> -like organisms 1269/76	Modified acid-fast stain	ELISA ^a		ELISA - antiserum <u>C.coli</u> 9BF2
			- rabbit antiserum to <u>Campylobacter</u> -like organisms 1269/76		
Initial sample					
284/86	+++	+++	2,560		<20
761/86	+++	+++	2,560		<20
<u>Mucosa 284/86^b</u>					
- unbound fraction from:					
WGA column	+++	+++	2,560		<20
Agarose column	+++	+++	2,560		80
GSII column	-	-	<20		<20
- washed fraction from:					
WGA column	+++	+++	640		<20
Agarose column	+++	+++	1,280		80
GSII column	-	-	<20		<20
- eluted fraction from:					
WGA column	+	+	320		40
Agarose column	+	+	<20		<20
GSII column	+	++	160		<20

a ELISA results expressed as reciprocal endpoint dilutions (see Chapter 4). Other antisera tested viz:
C.mucosalis 1248/72, C.hyointestinalis 124/73A⁴, C.jejuni 663/83 and "normal" rabbit serum 578/77 gave uniformly negative results (<20).

b Similar results obtained for mucosa 761/86.

+ - +++ Number of curved bacilli evident on staining or immunofluorescence, see legend to Table 4.

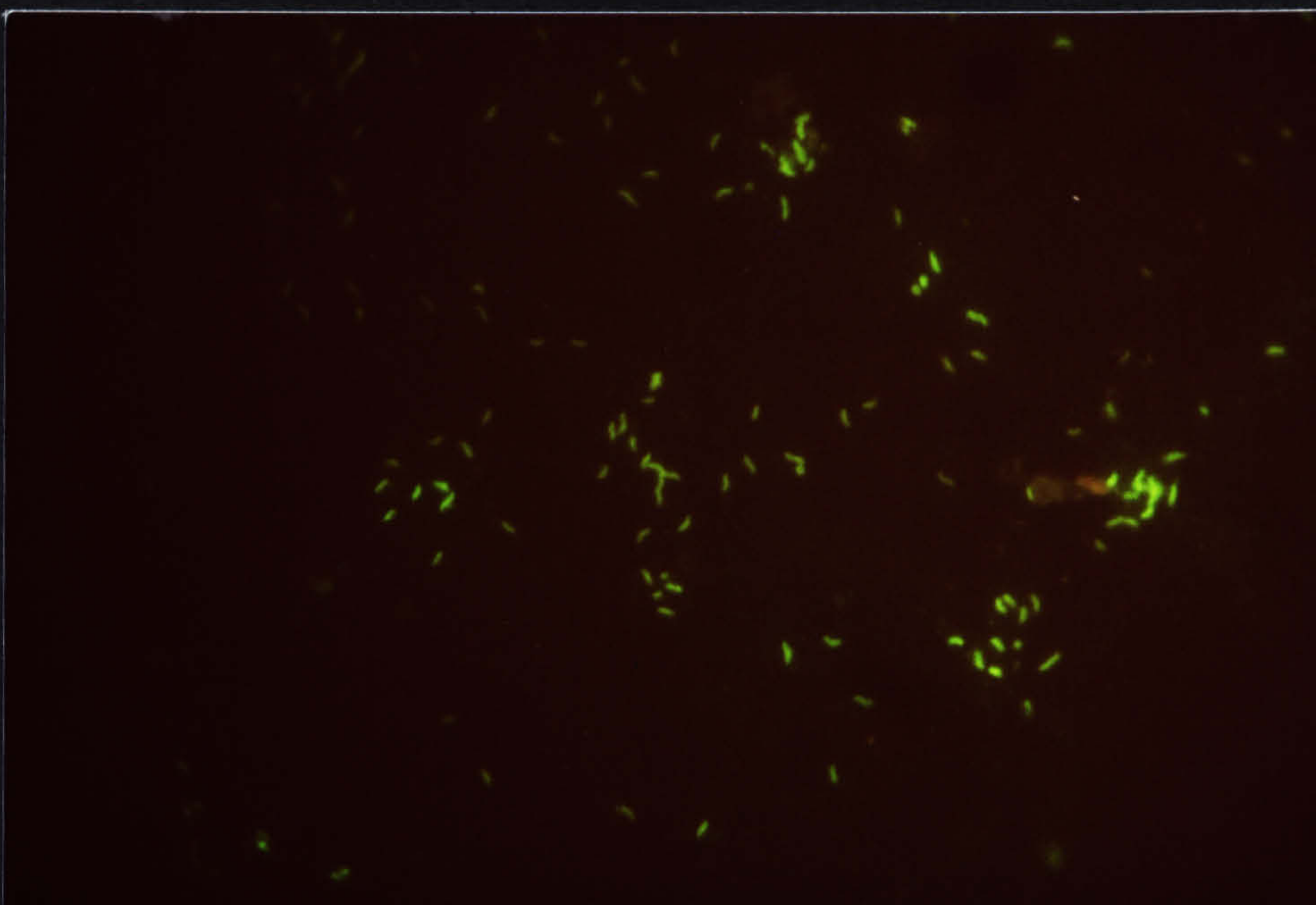
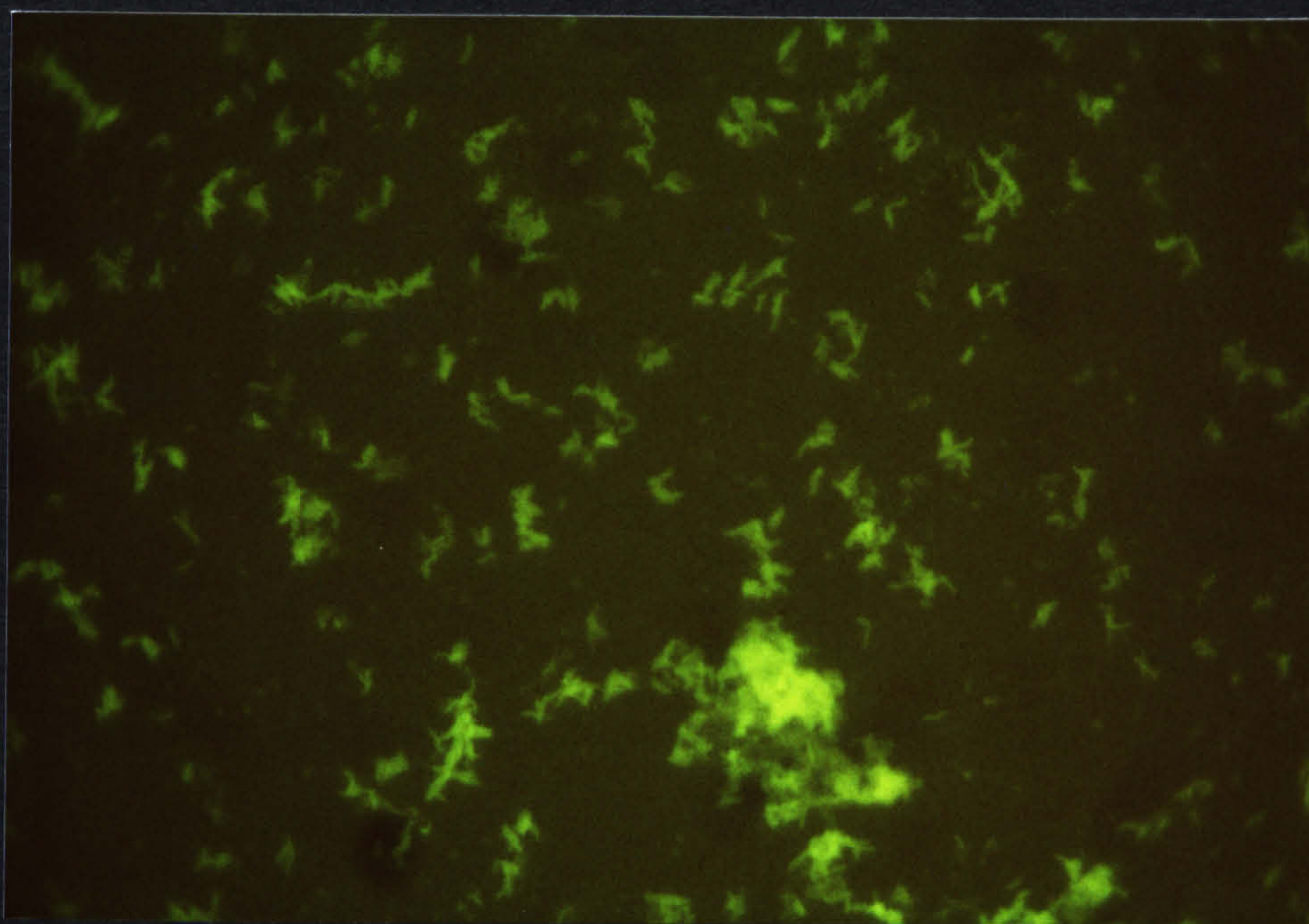
FIGURE 16.

A. Photomicrograph of smear of initial sample preparation of Campylobacter-like organisms partly purified from mucosa 284/86 by homogenization and filtration, prior to application to agarose columns. Indirect immunofluorescence assay with rabbit antiserum to Campylobacter-like organisms 1269/76, diluted 1:150 in phosphate-buffered saline, as the first antibody, and fluorescein-conjugated sheep anti-rabbit immunoglobulin as the second antibody. Brightly fluorescing curved bacilli, with some background material evident.

x 600.

B. Photomicrograph of smear of unbound fraction collected from a wheat-germ agglutinin-agarose column, following application of the initial sample preparation of Campylobacter-like organisms partly purified from mucosa 284/86. Indirect immunofluorescence assay as described above for A. Brightly fluorescing curved bacilli, with clear background.

x 600.



Polyacrylamide Gel Electrophoresis

The protein profiles of the Campylobacter spp, as well as the products of the mucosa 284/86 Campylobacter-like organisms after passage through a wheat-germ agglutinin-agarose column, are shown in Figure 17. The protein profiles of outer membrane protein and sonicated whole cell antigen preparations were essentially identical for the same bacteria with occasional minor proteins missing from the OMP profiles only. The profiles of C.jejuni and C.coli strains were dominated by a major outer membrane protein of 40K to 45K, with six to eight other prominent proteins. The profiles of C.mucosalis strains were dominated by a triplet of major outer membrane proteins between 50K and 65K, and four or five other prominent proteins. The C.hyointestinalis strain profiles had major outer membrane proteins at 45K and 50K, with three or four other prominent proteins.

Further preparations of Campylobacter-like organisms and normal pig intestine are presented in Figure 18. The protein profiles of the Campylobacter-like organisms extracted from porcine proliferative enteritis tissue were dominated by major structural proteins of 55K and 70K with two or three other prominent proteins between 50K and 80K. Minor components were recognised between 20K and 43K, including two distinct bands at 25K and 27K. The normal tissue 204/79 (not passed through a column) had minor components only, including ones of 26K and 70K.

Analysis of washed or eluted fractions from each of the various columns showed protein profiles of occasional minor proteins only. The protein profile of the unbound fraction from the agarose column was essentially the same as that from the WGA column, illustrated in Figure 17, lane 10, but with extensive background staining. The profile of the unbound fraction of the GSII-agarose column was of six to eight

FIGURE 17.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of preparations of Campylobacter spp and purified Campylobacter-like organisms. Gel stained with silver method of Morrissey (1981); molecular weights indicated, are expressed in thousands. Lanes: 1, outer membrane preparation of C.mucosalis 1248/72; 2, outer membrane preparation of C.mucosalis 124/73B4; 3, outer membrane preparation of C.hyointestinalis 124/73A4; 4, sonicated preparation of C.hyointestinalis 9AL3; 5, outer membrane preparation of C.jejuni 1268/84J; 6, sonicated preparation of C.jejuni 664/83; 7, sonicated preparation of C.coli 9BF2; 8, sonicated preparation of C.coli 9AF3a; 9, whole cell preparation of Campylobacter-like organisms purified from mucosa 284/86 by homogenization, filtration and passage through a wheat-germ agglutinin-agarose column; 10, sonicated preparation of Campylobacter-like organisms purified from mucosa 284/86 as described for lane 9.

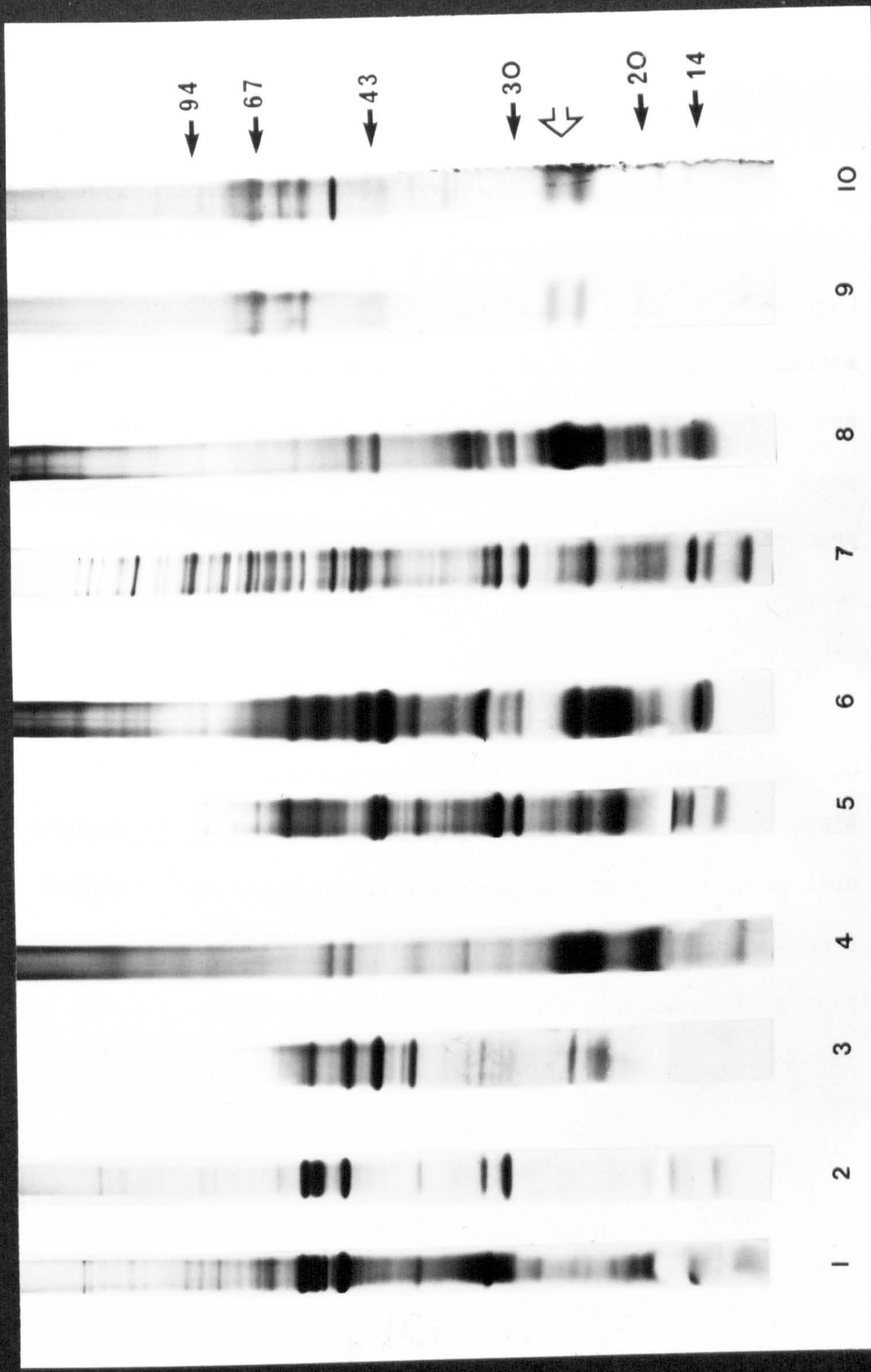
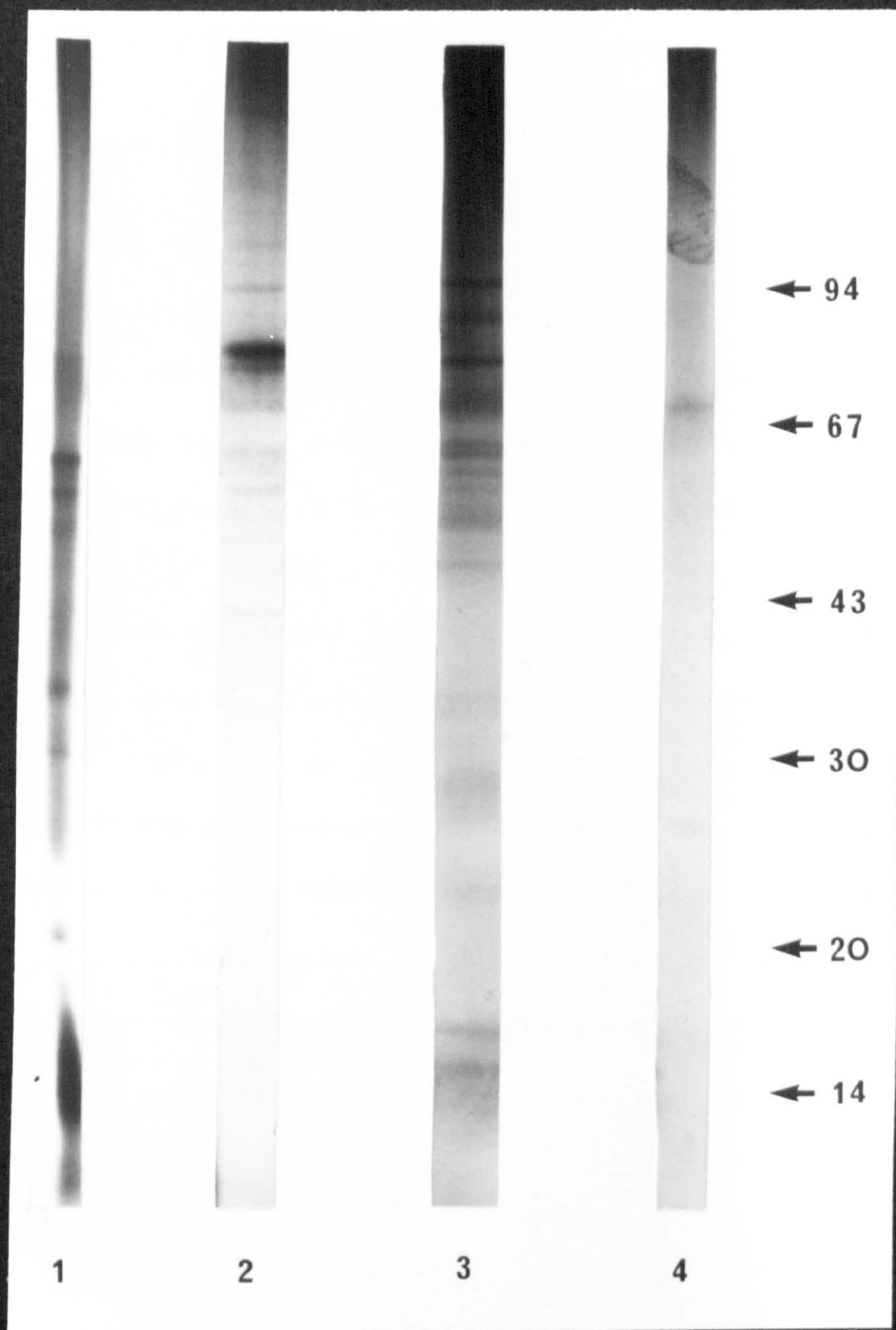


FIGURE 18.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of sonicated preparations of Campylobacter-like organisms and porcine intestinal material. Gel stained with silver method of Morrissey (1981); molecular weights indicated, are expressed in thousands. Lanes: 1, Campylobacter-like organisms partly purified from mucosa 761/86 by homogenization and filtration; 2, Campylobacter-like organisms purified from mucosa 761/86 by homogenization, filtration and passage through a wheat-germ agglutinin-agarose column; 3, Campylobacter-like organisms partly purified from mucosa 1269/76 by homogenization and filtration; 4, homogenate of mucosa 204/79.



minor proteins between 60K and 80K (not illustrated).

Electron microscopy of flagellin preparations showed small numbers of short flagellar segments. Analysis of these preparations on stained gels showed no distinct lines.

The protein profile of the C.coli 9BF2 strain with induced iron-deficiency, showed additional prominent proteins of 43K and 48K, when compared to profiles derived from the strain incubated in iron-containing medium.

Immunoblotting

Antibodies in the antiserum to Campylobacter-like organisms 1269/76 recognized the 25K and 27K components of Campylobacter-like organisms from mucosae 284/86 (Fig 19), 1269/76 and 761/86 (not illustrated). There was also recognition of the 55K and 43K proteins in some preparations of mucosa 284/86 (lane 2). Similar reactions were observed with antiserum 1080/76. These antisera only had minor reactions with components of C.mucosalis (21K, 45K, 55K, 60K), C.coli (43K, 60K, 62K) and the major outer membrane protein of C.jejuni (42K) with no other reactions (see Figure 19).

Antibodies in the C.jejuni 664/83 antiserum recognized the major outer membrane proteins of homologous and other strains of C.jejuni and C.coli. Numerous previous publications have illustrations of these reactions (e.g. Logan and Trust, 1983).

Similarly, antibodies in the two C.coli antisera recognized major outer membrane proteins of C.jejuni and C.coli strains. Homologous reactions of C.coli 9BF2, and its iron-deficient form are shown in Figure 20; the antiserum recognized the additional prominent proteins of 43K and 48K in the latter antigen preparation.

Rabbit antiserum to C.coli 9BF2 also recognized a 65K component of

FIGURE 19.

Immunoblot analysis of rabbit antiserum to Campylobacter-like organisms 1269/76 reacted against Campylobacter-like organisms and Campylobacter sp. Lanes: 1, sonicated preparation of Campylobacter-like organisms purified from mucosa 284/86 by homogenization, filtration and passage through a wheat-germ agglutinin-agarose column; 2, sonicated preparation of Campylobacter-like organisms partly purified from mucosa 284/86 by homogenization and filtration; 3, sonicated preparation of C.mucosalis 1248/72; 4, outer membrane preparation of C.jejuni 1268/84J; 5, sonicated preparation of C.coli 9BF2. Molecular weights indicated, are expressed in thousands.

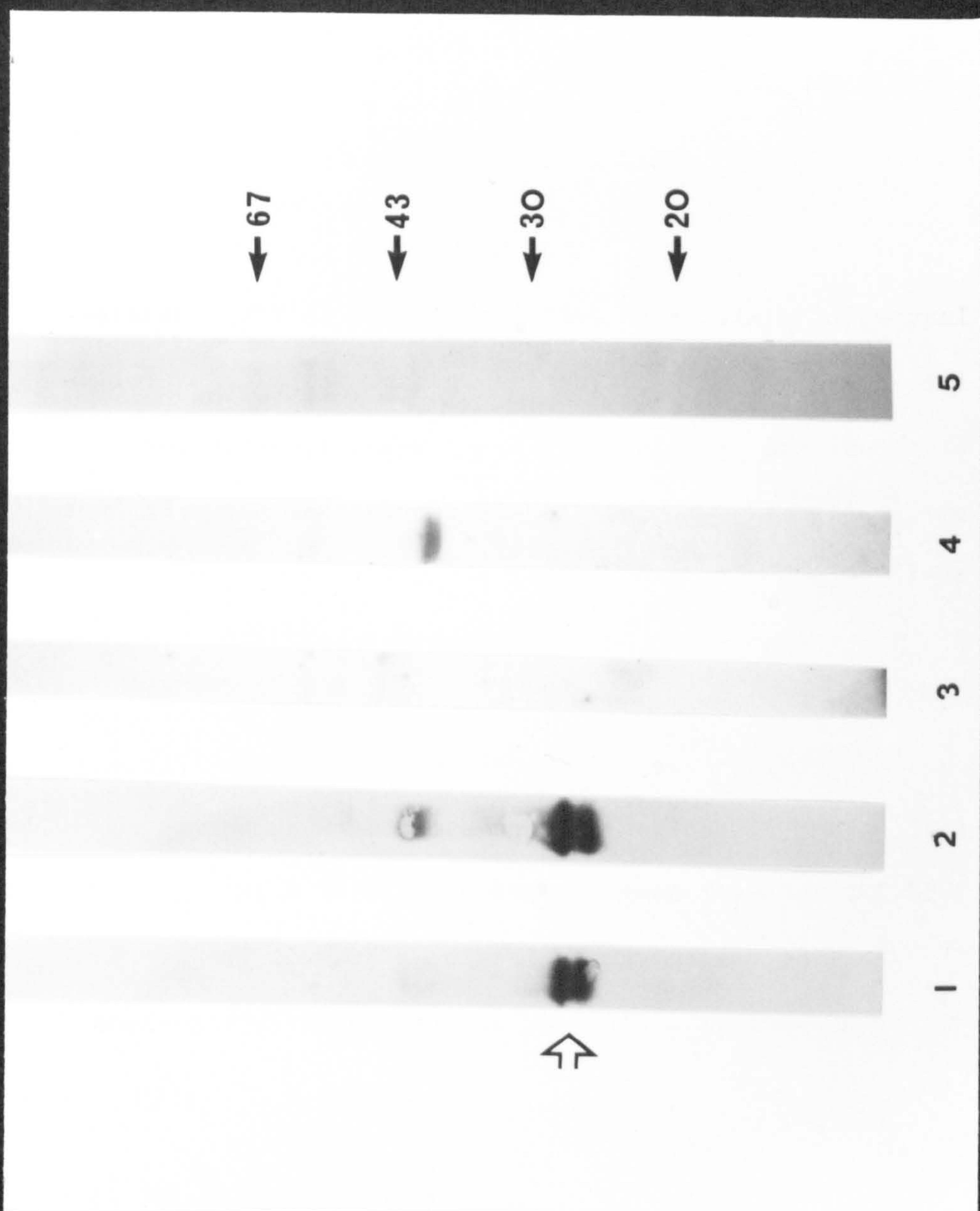


FIGURE 20.

Immunoblot analysis of rabbit antiserum to Campylobacter coli 9BF2 reacted against C.coli and Campylobacter-like organisms. Lanes: 1, sonicated preparation of C.coli 9BF2; 2, sonicated preparation of iron-deprived C.coli 9BF2; 3, sonicated preparation of Campylobacter-like organisms partly purified from mucosa 284/86 by homogenization and filtration.

Molecular weights indicated, are expressed in thousands.



1



2



3

← 94

← 67

← 43

← 30

← 20

mucosa 284/86 (Fig 20).

Antibodies in antisera to C.mucosalis 1248/72 and 124/73B4 recognized the outer membrane proteins of the C.mucosalis strains tested (Fig 21). These antisera also recognized a 60K protein in lanes containing the flagellin preparation of C.mucosalis 1248/72 and minor reactions were evident with proteins of other Campylobacter sp. Similarly C.hyointestinalis antisera 124/73A4 and 9AL3 recognized major outer membrane proteins of C.hyointestinalis strains (Fig 22) and a 60K flagellin protein of C.hyointestinalis 124/73A4 (lane 2). These antisera also recognized outer membrane proteins of C.mucosalis and C.jejuni strains (Fig 22).

No further reactions were evident, between any antisera to Campylobacter sp, and any mucosal preparation.

Rabbit antiserum 578/77 did not recognize any antigens tested.

Discussion

Intracellular Campylobacter-like organisms were purified from lesions of proliferative enteritis and compared in gel electrophoresis protein profiles and immunoblotting reactions to Campylobacter spp known to be associated with the disease. The intracellular organisms tested had a distinctive protein profile dissimilar to the profiles of the known Campylobacter spp. Furthermore, in immunoblotting reactions, each of the Campylobacter sp antisera reacted strongly with homologous antigens, but none reacted with Campylobacter-like organisms prepared from lesions, except for a minor reaction seen with one serum. Similarly antisera to Campylobacter-like organisms showed a strong reaction to 25K to 27K components of homologous antigens, with only minor reactions to various other components of the cultivated Campylobacter spp. The reactions of the antisera correlated with

FIGURE 21.

Immunoblot analysis of rabbit antiserum to C.mucosalis 1248/72 reacted against Campylobacter spp. Lanes: 1, outer membrane preparation of C.mucosalis 1248/72; 2, outer membrane preparation of C.mucosalis 124/73B4; 3, outer membrane preparation of C.jejuni 1268/84J; 4, sonicated preparation of C.coli 9BF2.

FIGURE 22.

Immunoblot analysis of rabbit antiserum to C.hyointestinalis 124/73A4 reacted against Campylobacter spp. Lanes: 1, outer membrane preparation of C.hyointestinalis 124/73A4; 2, flagellin preparation of C.hyointestinalis 124/73A4; 3, outer membrane preparation of C.mucosalis 1248/72; 4, outer membrane preparation of C.mucosalis 124/73B4; 5, outer membrane preparation of C.jejuni 1268/84J.

Molecular weights indicated, are expressed in thousands.



1



2



3



4

← 94

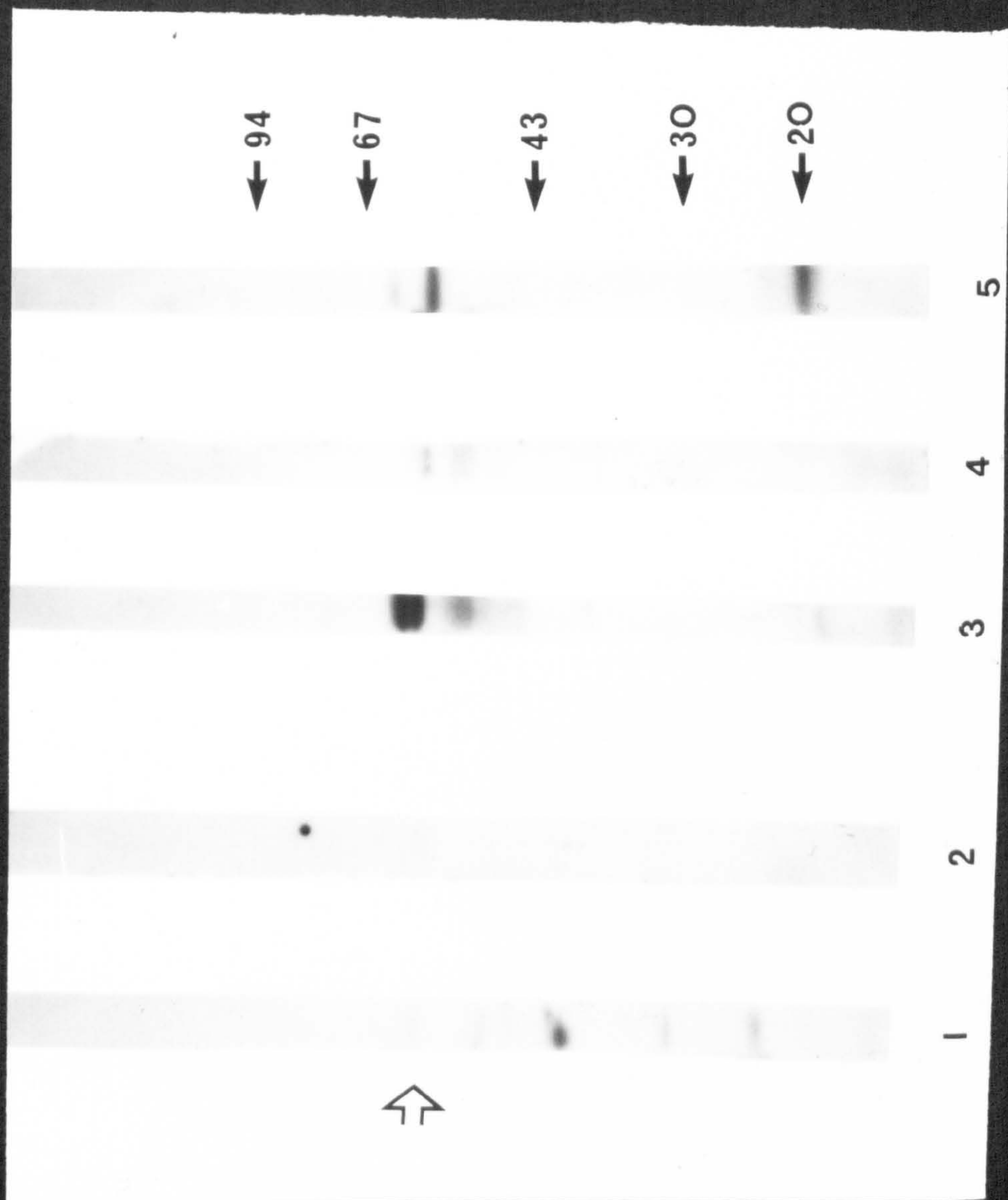
← 67

← 43

← 30

← 20

← 14



immunofluorescence and ELISA results with whole cell assays. Therefore it is likely that the intracellular Campylobacter-like organisms have a distinctive antigenic profile and that the 25 and 27K components are the major antigenic components detected in each assay. It is possible that the intracellular organisms are either a new, uncultured Campylobacter sp (with different antigens) or one (or more) of the known Campylobacter sp which undergoes a marked shift in size and antigenicity of their outer membrane components upon entry into enterocytes. The results of this Chapter suggest that the latter possibility is less likely to be true.

There was a wide range of lectin reactions among the antigens tested, similar to previous results with Campylobacter sp (Wong et al, 1985; Gill and Corbel, 1986). However C.mucosalis did not react with any lectin tested, indicating some outer membrane differences to the other Campylobacter sp. Wheat-germ agglutinin showed a consistent ability to bind to C.coli strains and to enterocyte components, in this and previous studies (Etzler and Branstrator, 1974; Wong et al, 1985). Analyses of the samples passed through columns containing wheat-germ agglutinin suggested that intracellular Campylobacter-like organisms could be purified from affected mucosae. These organisms showed similar immunofluorescence and ELISA reactions to those seen with filtered mucosal preparations (Chapter 4), but no background material was evident (Fig 16) and no Campylobacter sp could be cultured, although no Campylobacter sp was isolated from any agarose column fraction. Given the ability of GSII lectin to bind to Campylobacter-like organisms purified from lesions (Table 11) it was thought that these organisms may have been specifically purified by elution after being bound onto a GSII-column. However the purification obtained with this approach was poor, possibly because specific release of the

organisms did not occur during elution. Technical modification may improve the results of this approach. The preferred approach was one where the organisms of interest passed through the lectin column unbound, but likely contaminants were retarded.

Gel electrophoresis has been useful in analysing other bacteria purified from tissues (Lukehart et al, 1982). Campylobacter sp have been shown to have significant differences in their outer membrane protein profiles, reflecting valid genetic differences between species, except that C.jejuni and C.coli, which have nearly 30 per cent DNA homology, have similar profiles (Blaser et al, 1983; Hanna et al, 1983; Wenman et al, 1985). Therefore the apparently different protein profile of the purified Campylobacter-like organisms may reflect a valid difference between them and the Campylobacter spp tested. The use of either sonication or outer membrane preparation methods has previously been shown not to significantly alter Campylobacter sp profiles (Blaser et al, 1983). Flagellin is a highly conserved, 60K component of Campylobacter spp (Wenman et al, 1985). It is also a dominant immunogen, and can show cross-reactions with antibodies prepared against heterologous Campylobacter sp flagellin (Wenman et al, 1985; Newell, 1987). It was detected in each of the cultured Campylobacter sp tested here, but no 60K component was detected in the intracellular Campylobacter-like organisms.

Production of iron-deficiency in Campylobacter sp may cause iron chelating siderophores and separate membrane receptors to be produced on the bacteria (Field et al, 1986). Changes consistent with these processes were evident in the morphology (see Chapter 4) and the protein profiles of the strains tested. Iron-deficiency did not appear to induce products consistent with the intracellular Campylobacter-like organisms in any Campylobacter sp.

Immunoblotting has been widely used as a technique for detecting the antigenic components of Campylobacter spp. It has a greater sensitivity at detecting both protein and lipopolysaccharide components present in outer membranes of Campylobacter sp than gel electrophoresis alone (Nachamkin and Hart, 1985; Preston and Penner, 1987). A possible disadvantage of the technique is that bacterial components are boiled in reducing buffer prior to separation and reaction with antibodies. This may therefore cause an alteration or a deletion of antigenic sites. However, immunoprecipitation of Campylobacter sp with relevant antisera, without heat or chemical treatments, gave similar antigenic profiles to those seen with immunoblotting (Blaser et al, 1984). Also immunoblotting may be a more sensitive technique for Campylobacter sp antigens than immunoprecipitation (Logan and Trust, 1983) and can accurately detect species-specific antigenic profiles (Blaser et al, 1984). Therefore the detection of 25 and 27K components apparently specific to the intracellular Campylobacter-like organisms was considered a valid reflection of a major antigen on the outer membranes of these bacteria only. The nature and origin of these components were not clear. They stained relatively weakly with silver stains for proteins, and technical difficulties did not permit useful lipopolysaccharide or glycoprotein stains to be performed on gels (data not shown). It is possible that these antigens are an altered form of components present in one or more Campylobacter sp, however this appears unlikely because only minor reactions of various C.mucosalis and C.jejuni/coli antigens were observed with antisera to the intracellular organisms. The significance of these reactions is difficult to assess, as non-specific minor reactions may occur between polyclonal rabbit antisera, particularly if raised against such complex, crude antigens, and related bacterial components. Both

C.mucosalis and C.fetus have been suggested to undergo antigenic changes at times (Corbeil et al, 1973; Lawson et al, 1977), but those studies did not include immunoblotting. C.mucosalis has three recognized serotypes, indicative of some variation in its surface structures (Lawson et al, 1977); however the two strains analysed (representing serotypes I and III) showed a similar antigenic profile.

CHAPTER 6

MONOCLONAL ANTIBODIES AGAINST INTRACELLULAR CAMPYLOBACTER-LIKE ORGANISMS I. PREPARATION AND IMMUNOASSAYS

Introduction

The results of Chapter 5 suggested that rabbit antisera to intracellular Campylobacter-like organisms in lesions of porcine proliferative enteritis could be used to probe the relationships of these organisms to cultured Campylobacter sp. However polyclonal rabbit antisera have the disadvantages of low titre, heterogenous antibody sites, limited supply and irreproducibility, compared to monoclonal antibodies (Kohler, 1981).

Several monoclonal antibodies have been prepared against C.jejuni, C.coli and C.fetus (Kosunen et al, 1984; Newell, 1986a,b; Nachamkin and Hart, 1986; Kosunen and Hurme, 1986), recognizing flagellar and lipopolysaccharide components of the bacterial cell wall.

Perhaps more significantly, monoclonal antibodies have been used to probe the structures of unculturable bacteria purified directly from tissue, Mycobacterium leprae from armadillo liver and Treponema pallidum from rabbit testicles. Initial experiments led to specific monoclonal antibodies against the bacteria and/or the host tissue (Gillis and Buchanan, 1982; Lukehart, 1986). Later experiments with purer bacterial preparations produced more bacteria-directed antibodies, enabling significant information about their structure to be derived by various immuno-assays (Kolk et al, 1984; Lukehart, 1986).

Therefore monoclonal antibodies were developed against the intracellular Campylobacter-like organisms of porcine proliferative enteritis.

Materials and Methods

Monoclonal Antibody Preparation

Mouse immunization

BALB/c mice were immunized according to the schedules listed in Table 13. The preparation of each immunogen was described in Chapters 4 and 5 (see Tables 8 and 12). Briefly, mucosae 1269/76 and 284/86 were homogenized, trypsinized and filtered to release intracellular organisms (Appendix 5). Organisms from 284/86 were further purified by passage through a wheat-germ agglutinin-agarose (WGA) column with or without passage through a Griffonia simplicifolia II agglutinin-agarose (GSII) column. Each preparation was diluted to a standard suspension in PBS-formalin as described previously.

Immunized mice were bled regularly from a tail vein and sera tested by the screening assays described below.

On the day of each fusion, the mouse was killed, its spleen removed aseptically and treated as described below.

Cell culture

A mouse myeloma cell line designated NS0, from the cell culture collection of the Royal (Dick) School of Veterinary Studies, Edinburgh was used as the fusion partner. It had previously been established to be non-secretory, and to be sensitive to aminopterin. Sensitization had been achieved by growing the NS0 in dilutions up to 0.02mg per millilitre of 8-azoguanine and selecting viable subcultures for further use.

All cells were maintained in RPMI 1640 medium (Gibco, U.K.), enriched with 10 per cent v/v foetal calf serum and containing L-glutamine and penicillin/streptomycin at concentrations outlined in Appendix 4. This was designated enriched RPMI medium, and

TABLE 13: Mouse immunizations and fusions

Mouse designation	Immunogen ^a	No. of mice treated	Age (days)	Method of inoculation	Volume of immunogen given (ml)	Adjuvant ^b
1.	Mucosa 1269/76 (0.8µm filtrate)	3	90	SC	0.5	FCA
	- booster	3	164	IV	0.3	None
	- Fusion 1	1	168			
	Mucosa 284/86 (unbound fraction WGA column)	1	69	SC	0.2	FCA
	- booster	1	90	IP	0.3	None
2.	- booster	1	104	IP	0.2	None
	- booster	1	128	IV	0.1	None
	- Fusion 2	1	132			
	Mucosa 284/86 (eluted fraction WGA column)	1	69	SC	0.2	FCA
	- booster	1	90	IP	0.3	None
3.	- booster	1	104	IP	0.2	None
	- booster	1	128	IV	0.1	None
	- booster ^c	1	142	IV	0.2	None
	- Fusion 3	1	146			
	Mucosa 284/86 (eluted fraction GSII column)	1	46	SC	0.1	FCA
4.	- booster	1	86	SC	0.1	FCA

a Details of each immunogen given in text.

b Adjuvant emulsified with an equal volume of immunogen prior to inoculation (where used).

c Mucosa 284/86 (unbound fraction WGA column) given as immunogen.

SC - subcutaneous IV - intravenous IP - intraperitoneal FCA - Freund's complete adjuvant

modifications used will be indicated. Amphotericin was added occasionally at a concentration outlined in Appendix 4. In the week prior to a fusion, NSO cells were transferred to a new flask each day, and fresh enriched RPMI medium added, such that logarithmic growth of cells occurred. All cells were cultured at 37°C in a humid incubator filled with air with 5 per cent v/v carbon dioxide.

Feeder cells/medium

Mouse peritoneal macrophages - These were collected one day prior to use in fusion 1. Two 3 month old BALB/c mice were killed, and their peritoneal cavities flushed with 10ml of Hank's balanced salt solution (Gibco, U.K.). The fluids were withdrawn, pooled and diluted to 50ml with enriched RPMI medium, with added hypoxanthine-aminopterin-thymidine (HAT) and B-mercaptoethanol (see Appendix 4): This suspension was incubated overnight at 37°C in air with 5 per cent carbon dioxide.

Mixed thymocyte medium - A batch of this feeder medium was prepared for fusion 2 and 3, by the method of Reading (1982). Briefly, a cell suspension of thymus from two rats (different breeds) was washed and suspended in enriched RPMI medium for 48 hours. The supernatant fluid was collected, filtered through a 0.22µm filter and stored frozen. Prior to use the medium was warmed to 37°C.

Cell fusion with polyethylene glycol

Fusion 1 - Mouse spleen cells and NSO cells were suspended in RPMI 1640 medium without foetal calf serum, washed once with this medium, counted separately, then combined in approximately equal numbers in a 50ml conical tube. Counts of spleen cells (white cell stain, Appendix 3) and NSO cells (trypan blue, see Appendix 3) are given in Table 14. Nine millilitres of spleen cells were combined with

TABLE 14: Cell fusions

Fusion Number	Cell type	Concentration of cells in suspension ^a (expressed as log ₁₀ ml ⁻¹)	Viability ^a (% of total cells)
1	myeloma	7.2	83
	spleen	8.2	99
	myeloma/fused	6.0	-
2	myeloma	7.2	92
	spleen	6.9	98
	myeloma/fused	6.3	-
3	myeloma	6.4	99
	spleen	7.1	100
	myeloma/fused	6.3	-

a Myeloma and spleen cells counted and viability assessed after staining (see Appendix 3) and microscopic examination in a haematocytometer. Fused cells were counted unstained.

10ml of myeloma cells. After centrifugation (700g, 5 minutes) the supernatant fluid was removed and the layered pellet mixed slowly with 0.7ml of polyethylene glycol (MW 4,000) buffered to pH8.5 (see Appendix 4) adding 0.01ml at a time from a pipette.

After incubation at 37°C for 3 minutes, 20ml of RPMI 1640 medium was added 0.1ml at a time. After centrifugation (1,000g, 5 minutes), the supernatant fluid was removed and the fused pellet resuspended in enriched RPMI medium, further enriched with foetal calf serum (final concentration 20 per cent v/v), HAT and B-mercaptoethanol (see Appendix 4). After counting, see Table 14, fused cells were distributed in 0.1ml amounts, containing 1.0×10^5 cells, to wells of tissue culture plates (Nunc, Denmark), already containing 0.1ml of the mouse peritoneal macrophage suspension. The remaining one millilitre of spleen cells was cultured in separate wells, as a residual antibody control.

Fusions 2 and 3 - Mouse spleen cells and NSO cells were suspended in RPMI 1640 medium without foetal calf serum, washed once with this medium, counted and combined as described above (see Table 14). After centrifugation (800g, 5 minutes), the supernatant fluid was removed and the layered pellet was loosened by tapping. One millilitre of the polyethylene glycol solution was layered on top of the pellet, then the tube was gently swirled for 8 minutes. After centrifugation (400g, 5 minutes), 5ml of RPMI medium, without foetal calf serum, was carefully layered on top of the solution, over a period of 2 minutes. The tube was gently swirled for 4 minutes to resuspend the cells. After centrifugation (700g, 5 minutes), the supernatant fluid was removed and enriched RPMI medium further enriched with foetal calf serum (final concentration 20 per cent v/v) HAT and B-mercaptoethanol was layered on top of the fused pellet and left to stand for 7 minutes. The cells

were resuspended by gently swirling the tube, then a further 20ml of this medium added. After counting, see Table 14, fused cells were distributed in 0.1ml amounts containing 2.5×10^5 cells, to wells of tissue culture plates, already containing 0.1ml of the mixed thymocyte medium. Spleen cells were cultured as for fusion 1.

Hybridoma culture

After one week, 0.05ml of enriched RPMI medium further enriched with foetal calf serum (final concentration 20 per cent v/v) and HAT was added to each well. All wells in fusion 2 were contaminated with fungal cultures and discarded. After two weeks for each remaining fusion, 0.1ml of supernatant fluid was removed from wells containing growing hybridoma cells, and tested by the screening assays (see below) for specific antibody. Hybridomas in wells of interest were expanded in numbers into separate tissue culture flasks (Nunc, Denmark). Aliquots of 10^6 cells were combined with a cell freezing mixture (see Appendix 4) cooled to -70°C over 2 days then transferred to liquid nitrogen.

Each hybridoma of interest was cloned twice in soft agar. For cloning, doubling dilutions of each cell line (one millilitre) were mixed with one millilitre of agar medium (0.5 per cent agarose in enriched RPMI medium, see Appendix 4) to make sloppy agar and pipetted on top of a prepared base of 15ml of the agar medium in separate petri dishes (90mm diameter). After one to two weeks incubation at 37°C in air with 5 per cent carbon dioxide, separate single-cell derived clones were pipetted into fresh enriched RPMI medium in tissue culture plates. Cloned cell lines were expanded and frozen as described above. During expansion of cell lines the concentration of foetal calf serum in the medium was reduced to 10 per cent, aminopterin was removed from the

medium, and then the remaining hypoxanthine-thymidine addition was removed.

Supernatant fluid was removed from expanded cell lines at least twice weekly and tested for titre and specificity by the screening assays (see below). Isotyping of immunoglobulins produced by cloned cell lines was performed with a commercial gel-diffusion kit (Serotec Ltd., U.K.). Briefly, 0.075ml of supernatant fluid was added to a central well, and 0.010ml of separate antisera to each murine immunoglobulin class added to surrounding wells. Visible precipitin lines readily developed after overnight incubation of the gel at 20°C.

Hybridoma growth in mice

BALB/c mice were injected intraperitoneally with 0.5ml of Pristane (2, 6, 20, 14-tetramethylpentadecane, Sigma Chemical Co., U.K.) and 10 days later with 5×10^6 cells from one of each cloned cell line. Ascitic fluid from the growth of each intraperitoneal hybridoma was collected 5 to 14 days later, and tested for titre and specificity by the screening assays (see below).

Screening assays

Enzyme immunoassay - Wells of ELISA plates were passively adsorbed with 0.05ml of a 1:50 dilution in PBS of Campylobacter-like organisms 1269/76, as described in Chapter 4. Either 0.05ml of mouse serum diluted 1:10 in PBS, or neat supernatant fluid was added as the first antibody, and peroxidase-conjugated rabbit anti-mouse antiserum added as the second antibody as part of the ELISA described by Voller et al (1980), see Chapter 4.

Positive mouse sera or cell lines were further tested for titre and specificity of their antibodies. Doubling dilutions of serum, supernatant fluid or ascitic fluid in ELISA dilution buffer (see

Appendix 1) were reacted in wells adsorbed with one of each of the porcine intestinal materials or Campylobacter sp antigen preparations described in Chapter 4. Positive serum from immunized mice, and pooled normal mouse serum were used as controls in each assay. The dilution giving an absorbance of > 0.200 was taken as the endpoint.

Indirect immunofluorescence assay - Glass slides were coated with Campylobacter-like organisms from mucosa 1269/76 as described in Chapter 4. 0.01ml of supernatant fluid was added as first antibody and fluorescein conjugated sheep anti-mouse antiserum added as the second antibody and the slides examined as described in Chapter 4.

For titre and specificity, doubling dilutions in PBS of each fluid were reacted with each of the antigen preparations described in Chapter 4. Positive and negative controls were those given above. The final dilution giving brightly fluorescing material was taken as the endpoint.

Immunoblotting

Each of the Campylobacter sp, Campylobacter-like organism and porcine intestinal material antigen preparations described in Chapter 5 was solubilized, separated by gel electrophoresis and transferred to nitrocellulose paper as described in that Chapter. After non-specific binding was blocked (Blocking buffer, see Appendix 1), the papers were incubated at 20°C for 18 hours in each positive supernatant fluid. In some cases the immunoglobulins in 200ml of each fluid were concentrated to a 20ml final volume of solution, by the use of protein A-Sepharose affinity chromatography (Kohler, 1981; see Appendix 5 for method). Separate incubations were also made with supernatant fluid from hybridomas producing IgG₃ or IgM antibodies from fusions in mice immunized with irrelevant antigens (Trypanosoma sp). After washing,

papers were incubated in iodine¹²⁵ conjugated goat anti-mouse immunoglobulin (fusion 1) or in peroxidase-conjugated rabbit anti-mouse immunoglobulin serum (fusion 3) diluted 1:2,000 in blocking buffer and processed as described in Chapter 4.

Antigen Recognition

Clarification of the nature of the antigen recognized by positive monoclonal antibodies from fusion 3 was attempted. Campylobacter-like organisms from mucosa 1269/76 were air-dried onto glass slides, washed 3 times in PBS and incubated with Protease XXV (Sigma Chemical Co., U.K.) in concentrations of 0.01, 0.05 or 0.1 per cent w/v in PBS for 30 minutes at 37°C. Both protease-treated and non-treated controls were then washed, incubated with positive supernatant fluid and processed for indirect immunofluorescence (as described above).

To clarify if the antigen recognized by antibodies from fusion 3 was present on all the predominant Campylobacter-like organisms extracted from an affected mucosa, double antibody staining was performed. Campylobacter-like organisms from mucosa 1269/76 were air-dried onto glass slides, incubated with each positive supernatant fluid for 30 minutes at 37°C, washed in PBS, incubated with fluorescein-conjugated sheep anti-mouse IgG serum for 30 minutes at 37°C then washed in PBS. Slides were then incubated with a 1:150 dilution of rabbit antiserum 1269/76 (see Chapter 4) in PBS for 30 minutes at 37°C, washed in PBS, incubated with rhodamine-conjugated goat anti-rabbit IgG serum for 30 minutes at 37°C, then washed in PBS. Appropriate controls were incorporated. Slides were examined under a fluorescence microscope, with appropriate filters.

Results

Reciprocal endpoint dilutions of the sera of the four immunized

mice were 100, 1,000, 1,000 and 5 for mice 1 to 4 respectively (see Table 13) in the ELISA, and 5, 200, 200 and 5 in the immunofluorescence assay against antigen 1269/76 when tested at the time of the last booster dose. No reactions were observed in either assay when normal mouse sera were tested.

The results of screening hybridoma supernatant fluid is given in Table 15. A typical positive immunofluorescence reaction is shown in Figure 23. Identical titres were observed with Campylobacter-like organisms from mucosas 284/86 and 761/86. No reactions were observed with normal mucosa 204/79 or with Campylobacter sp antigen preparations except for occasional absorbances in ELISA of >0.2 in wells coated with C.mucosalis or C.hyointestinalis antigen and reacted with neat supernatant fluid from cell line SIG3.

Mice injected with SIG3 or SIH93 cell lines did not produce ascitic fluids. Antibodies in the supernatant fluids SIG3, SIH93 and S3CII from fusion 1 all apparently recognized two or three outer membrane proteins of C.jejuni, and SIG3 also recognized a 50K protein of C.mucosalis (Fig 24). None of these recognized any component of other antigens, including Campylobacter-like organisms or normal porcine intestine, in immunoblotting, despite showing reactions in ELISA.

Antibodies in the supernatant fluids IG4 and 4F5 recognized the 25K to 27K component of Campylobacter-like organisms from mucosae 284/86, 1269/76 and 761/86 (Fig 25). These antibodies were the only ones to react with these organisms in both immunofluorescence and ELISA assays. No reaction was observed between these antibodies and cultured Campylobacter sp antigen or normal mucosa 204/79. Also no reaction was evident between IgM or IgG₃ antibodies to irrelevant antigens and any Campylobacter sp or porcine intestinal antigens.

TABLE 15: Hybridomas

	Fusion 1			Fusion 3	
<hr/>					
<u>No. of wells</u>					
Seeded with fused cells	288			234	
Hybridomas present after 2 week culture	40 (13.9%)			65 (27.8%)	
Positive ELISA ^a	3 (1%)			2 (0.85%)	
Positive IFA ^a	0			2 (0.85%)	
 <u>Clones</u>					
Designation	SIG3	S3CII	SIH93	IG4	4F5
Isotype	IgM	IgM	IgM	IgG ₃	IgG ₃
 ELISA Titre					
- supernatant fluid	5	1	1	80	80
- ascites	N.A.	640	N.A.	2,560	2,560
 IFA titre					
- supernatant fluid	0	0	0	20	20
- ascites	N.A.	0	N.A.	1,280	1,280

a IFA - indirect immunofluorescence assay

ELISA - enzyme-linked immunosorbent assay. Titres given as reciprocal endpoint dilution giving OD > 0.2 (ELISA) or brightly fluorescing bacteria (IFA) against antigen preparation of Campylobacter-like organisms 1269/76.

N.A. Not available.

FIGURE 23.

Photomicrograph of smear of antigen preparation of Campylobacter-like organisms partly purified from mucosa 1269/76 by homogenization and filtration. Indirect immunofluorescence assay, with monoclonal antibody 4F5 in neat supernatant fluid, as the first antibody, and fluorescein-conjugated sheep anti-mouse immunoglobulin as the second antibody. Brightly fluorescing curved bacilli evident.

x 600.

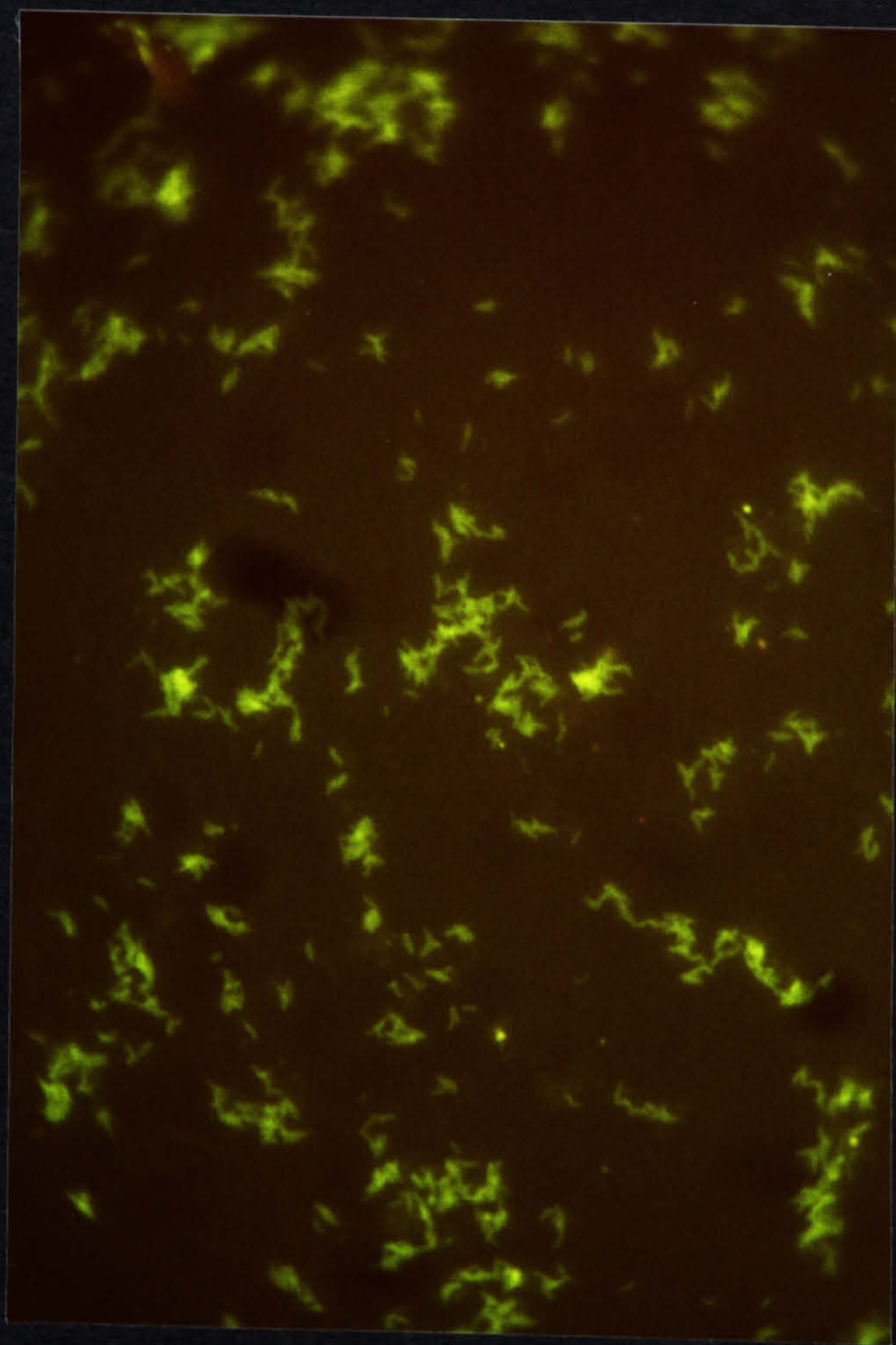
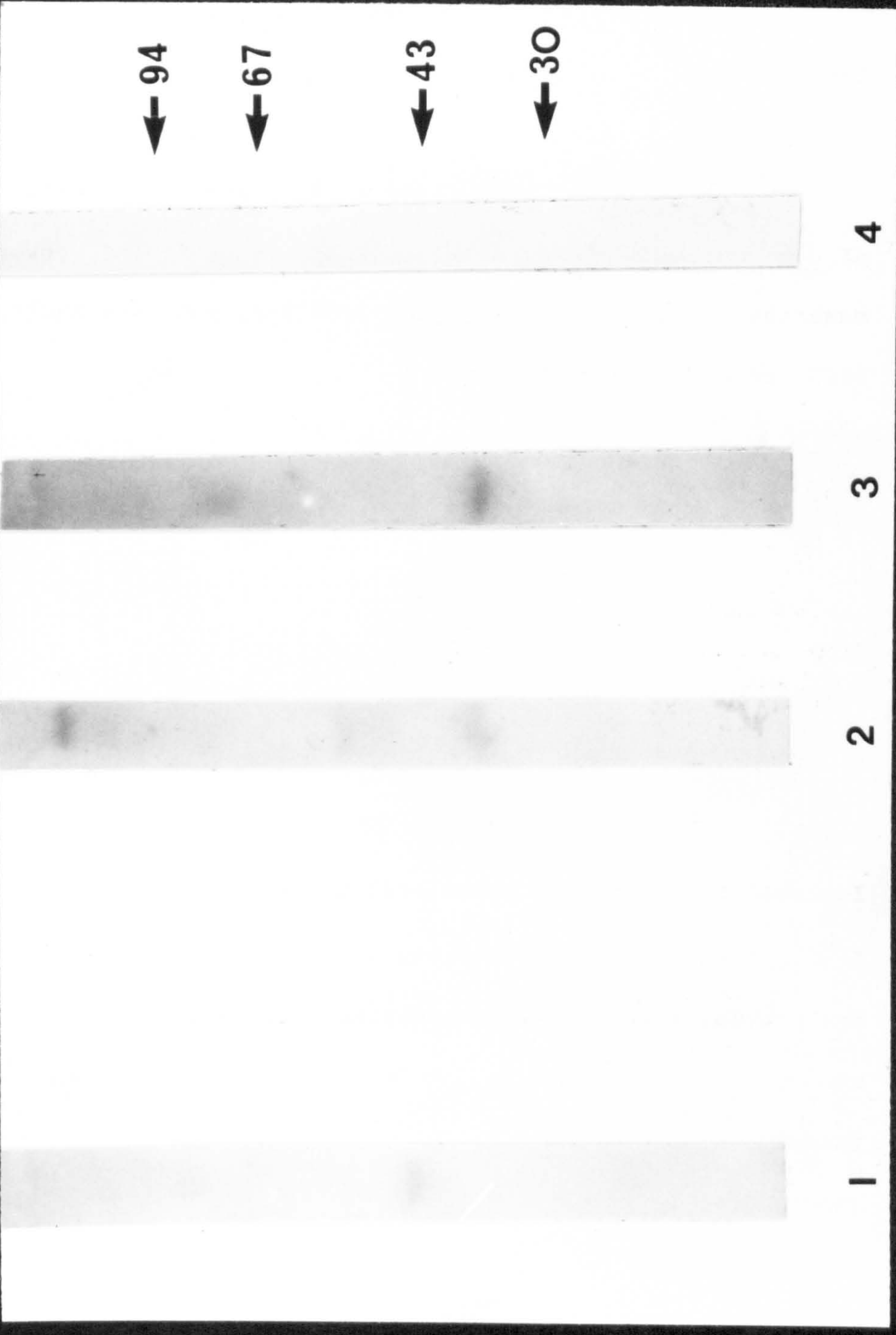


FIGURE 24.

Immunoblot analysis of monoclonal antibodies in supernatant fluids from hybridomas produced in fusion 1, reacted against Campylobacter spp. Lane 1, sonicated preparation of C.mucosalis 1248/72, antibody SIG3, Lanes 2 to 4, outer membrane preparation of C.jejuni 1268/84J, antibody S3CII, SIG3 and SIH93 respectively.

FIGURE 25.

Immunoblot analysis of monoclonal antibodies in supernatant fluids from hybridomas produced in fusion 3, reacted against Campylobacter-like organisms partly purified from mucosae by homogenization and filtration. Lanes 1 and 2, mucosa 284/86, antibody 4F5 and IG4 respectively, lane 3, mucosa 1269/76, antibody IG4, lane 4, mucosa 761/86. Molecular weights indicated, are expressed in thousands.





1



2



3



4

← 67

← 43

← 30

← 20

Incubation of Campylobacter-like organism antigens with protease had no observable effect on immunofluorescence reactions with antibodies IG4 or 4F5.

Double antibody staining of Campylobacter-like organisms showed all organisms on each slide apparently stained by antibodies IG4 or 4F5, with no additional organisms detected by a subsequent rabbit antiserum reaction.

Discussion

Monoclonal antibodies were produced in fusion 3 that were apparently specific to the intracellular Campylobacter-like organisms in the lesions of proliferative enteritis. Immunoblotting results showed that these antibodies only bound to a 25K to 27K outer membrane component present in the intracellular organisms. Reactions with this component could not be detected in assays with normal pig intestine, or Campylobacter sp antigen. Therefore the intracellular organisms are distinct from currently recognized Campylobacter sp. Similar conclusions were made with rabbit antisera reactions (Chapters 4 and 5), but the use of monoclonal antibodies makes cross-reactions with unrelated antigenic sites much less likely.

Specific antibodies were only produced in fusion 2 and 3, after the mice were immunized with affinity-purified antigen. In comparison, fusion 1 produced antibodies of a non-specific character, which showed confusing cross-reactions in ELISA. The reasons for these cross-reactions could include use of crude immunogen and a short immunization schedule, leading to IgM antibodies of poor specificity for the bacterial component of the immunogen. Also, insufficient emphasis was placed in this first fusion on the importance of having positive reactions in both ELISA and immunofluorescence assays, before

hybridomas were selected. All of these technical problems with fusions involving tissue-derived bacterial antigen, have been noted in studies with Mycobacterium leprae and Treponema pallidum (Kolk et al, 1984; Engers et al, 1985; Lukehart, 1986). IgM monoclonal antibodies are more likely to be involved in non-specific reactions than are IgG antibodies (Ghosh and Campbell, 1986). The use of whole bacteria, carefully purified from cellular material has been associated with relative success in producing specific monoclonal antibodies, in this and previous studies (Kolk et al, 1984; Lukehart, 1986).

The nature of the distinctive 25K to 27K components in the intracellular Campylobacter-like organisms was apparently not completely protein, as its antigenic site resisted protease treatment. However, it may be a glycoprotein or similarly mixed component, with protection of the antigenic site. The presence of twin components of 25K to 27K, suggests a minor change in molecular structure, such as addition of glycosyl groups, in one basic component. Twin components of 60K to 62K have been seen with reaction of monoclonal antibodies to C.jejuni (Newell, 1986a). The role of the 25K to 27K component is not clear, but immunofluorescence results suggest it is a major structural component of the outer membrane of the intracellular organisms. No evidence was detected of this component being pig tissue antigen.

Probing of the intracellular Campylobacter-like organisms in proliferative enteritis in hamsters, with monoclonal antibodies to Campylobacter sp (Stills et al, 1987), also reached the conclusion that these organisms were distinct from recognized Campylobacter sp. However their study did not include antibodies specific to the intracellular organisms. Monoclonal antibodies previously prepared against C.jejuni, C.coli, C.fetus or C.pyloridis have confirmed their ability to distinguish between Campylobacter sp, and to bind to various

components of the outer membranes of the organisms (Kosunen and Hurme, 1986; Engstrand et al, 1986). In one study, most monoclonal antibodies produced against C.jejuni, were directed against lipopolysaccharide components of the outer membranes (Kosunen and Hurme, 1986).

Also, monoclonal antibodies to either M.leprae or to Campylobacter sp have been found to be effective in immunoblotting methods in identifying the relevant antigen within the organisms after separation by gel electrophoresis (Kolk et al, 1984; Engstrand et al, 1986). Therefore the conclusions that there is a 25K to 27K component in the intracellular Campylobacter-like organisms, that is distinctive and involved in positive immunoassays, are likely to be valid. It is surprising that monoclonal antibodies only appeared against these components, mimicing the results obtained with rabbit antisera. It may have been expected that there would have been some variation in the sites and specificities recognized by a number of monoclonal antibodies produced for a single immunogen. Only two useful monoclonal antibodies were produced and further studies may reveal variations.

CHAPTER 7

MONOCLONAL ANTIBODIES AGAINST INTRACELLULAR CAMPYLOBACTER-LIKE ORGANISMS

II. USE IN EVALUATION OF PATHOGENESIS OF PROLIFERATIVE ENTEROPATHY

Introduction

Results in previous Chapters have indicated that the intracellular Campylobacter-like organisms are antigenically distinct from cultured Campylobacter spp. A previous study (Lawson et al, 1985) indicated that rabbit antisera prepared against preparations of the intracellular organisms reacted with a component(s) present on the intracellular organisms within various lesions of proliferative enteropathy in both pigs and hamsters. However the use of polyclonal reagents and the possibility that some rabbits may carry "natural" antibody to the intracellular organisms means that no firm conclusion could be made about the organisms in various lesions being identical. Therefore, the intracellular organisms within various lesions required further examination with the monoclonal antibody reagents.

The clear understanding of the pathogenesis of proliferative enteropathy is partly obscured by the occurrence in some lesions of a variety of Campylobacter sp in the lumen of the intestine, while there are also enterocytes parasitised by intracellular organisms (see Chapter 2). In the developed lesions of proliferative enteritis, there are generally no bacteria evident in the crypt lumina or associated with the mucosa (Rowland and Lawson, 1974). In animals with early or developing lesions, it may be possible that organisms are entering the enterocyte cytoplasm from the crypt lumen. Identification of such organisms either undergoing endocytosis or closely associated with the mucosa would be of significance in establishing the pathogenesis of proliferative enteritis. The examination of these organisms by in situ immunological assays on sections, incorporating relevant antisera could provide information on the relationships involved.

Detailed probing of sections of lesions is possible by immunogold electron microscopy. This technique does not always work, but has been

used to examine the sites within bacteria that are recognized by specific antibodies (Beesley et al, 1982). A variety of techniques were tested to optimize the likelihood of useful results.

The possibility that the monoclonal antibodies could be used to detect organisms specific to the disease in the faeces of pigs was also tested, to provide information on the possible excretion of such organisms during the disease or at other times. Such excretion could be of epidemiological and diagnostic significance.

Materials and Methods

Indirect Immunofluorescence Assay of Intestinal Sections

Methods used

Five μ m sections of each tissue listed below, were digested by trypsin, then washed in PBS as previously described (Lawson et al, 1985). Neat supernatant fluid containing monoclonal antibody IG4 or 4F5 (see Chapter 6) was added as the first antibody, and incubated for 30 minutes at 37°C. After washing, fluorescein-conjugated sheep anti-mouse serum was added as the second antibody, and incubated for 30 minutes at 37°C. After washing, sections were examined under a fluorescence microscope. Histological sections were available for each tissue.

Porcine intestine sections

The origin of the intestines examined and the form of any histologically confirmed lesions are given with the Results in Table 16.

Hamster intestine sections

Intestines were obtained from three sources. First, the 16 hamsters in groups 12 and 13 of Chapter 3 (see Table 6), and the

hamsters in the control group 18 from the same experiment. Second, two hamsters naturally affected with proliferative enteritis, and a normal hamster from the same Institute (kind donation of Dr. J. Fox, Massachusetts Institute of Technology, U.S.A.). Third, three hamsters, designated 56/80, 57/80 and 58/80, which had been dosed orally with homogenised intestinal mucosa from a naturally-affected hamster, and killed 7, 14 and 21 days after infection respectively. Histological lesions of proliferative enteritis were evident in 57/80 and 58/80. Silver stains showed intracytoplasmic curved bacilli were present in the enterocytes in the sections of all three intestines. A control hamster, designated 55/80, from the same experiment was also examined. These tissues were a kind donation of M. La Regina, St. Louis University School of Medicine, U.S.A. who previously published experimental details (Regina and Lonigro, 1982).

Immunogold Staining of Intestinal Sections

Methods used

Etching - Fifty nm sections of glutaraldehyde-fixed intestine were prepared as described in Chapter 2, and placed on nickel-gold grids. Three etching techniques were used in various combinations on these sections, prior to application of antibodies (Smart and Millard, 1983). First (Deresin), some sections were immersed in a 1:1 mixture of ethanol and propylene oxide, with excess sodium hydroxide, for one minute at 20°C. Second (Deosmicate), some sections were immersed in 0.5 per cent v/v periodic acid for 15 minutes at 20°C. Third (Trypsinise), some sections were immersed in 0.1 per cent w/v trypsin, with 0.2 per cent w/v calcium chloride in water (pH 7.8), for 10 minutes at 37°C. The combinations used on separate sections were deresin, deosmicate with or without trypsinising; deosmicate with or without

trypsinising and untreated. All sections were then washed in tap water for 30 minutes, rinsed in distilled water, then washed in a Tris-albumin buffer recommended by the manufacturers of gold suspension (BSA-Tris, see Appendix 1; Janssen Biotech, Belgium).

Immunogold assay - Sections were immersed in neat supernatant fluid from hybridoma IG4 (see Chapter 6), for 30 minutes at 37°C. After washing in BSA-Tris, sections were immersed in a 1:100 dilution of colloidal gold particles (5 or 15nm diameter) coated with Staphylococcus aureus protein A (Auroprobe EM, Janssen Biotech, Belgium) in BSA-Tris, for one hour at 37°C. After washing in BSA-Tris, then distilled water, sections were stained with uranyl acetate/lead citrate (see Chapter 2). Other sections were treated as above, but without the addition of supernatant fluid IG4.

Hamster and porcine intestine sections

Portions of ileum and large intestine from pigs 1 and 2 in Chapter 2, were examined. Portions of ileum and large intestine from a hamster, designated 111/82, with experimentally-induced proliferative enteritis were also examined. This hamster had been necropsied 21 days after infection as part of a separate study conducted by Dr. G.H.K. Lawson.

Indirect Immunofluorescence Assay of Faeces

Methods used

Faeces collected from pigs were diluted 1:5 in PBS containing 0.3 per cent v/v formalin, and a sample from each air-dried onto glass slides. Samples were fixed in acetone for one minute, dried, then supernatant fluid or ascitic fluid (diluted 1:200 in PBS) from hybridoma IG4 or 4F5 (see Chapter 6) was added as the first antibody. Incubation, washing and second antibody steps were as described

previously.

Hamster and porcine faeces

Faeces collected at necropsy of the eight pigs in Chapter 2, the eight hamsters in group 13, and four control hamsters in group 18 of Chapter 3 (see Table 6) were examined. Also three pigs, 5 to 9 months old, from a local farm (IAPGR, see Table 16) were noticed to be clinically ill, with severe haemorrhagic diarrhoea. Faeces were collected, and two to seven days later each pig died despite treatment. Necropsy of these pigs revealed gross and histological lesions of proliferative haemorrhagic enteropathy.

Subsequent to these clinical cases, sixteen 5 week old pigs on this farm were identified at weaning, and their faeces examined at least every 2 weeks, until they were 6 months old. These faeces (n =154) were also diluted 1:5 in PBS and a swab of each diluted sample smeared onto Skirrow's agar (Skirrow, 1977) and RNBGT agar (McCartney et al, 1984), and cultured for Campylobacter spp as described previously.

Results

Indirect Immunofluorescence Assay of Intestinal Sections

The results of examination of porcine intestines are summarised in Table 16. Sections with 3+ immunofluorescence showed brightly fluorescing curved bacilli within the cytoplasm of enterocytes lining the crypts of proliferative mucosa (Fig 26), and brightly fluorescing globular bodies within mucosal mononuclear cells. Pig 763/86, see Table 16, had a fewer number of brightly fluorescing bacilli evident within affected mucosa. Fluorescent material was not detected within any crypt lumen.

All of the hamsters that had histologically confirmed

TABLE 16: Immunofluorescence of porcine intestinal sections with monoclonal antibody raised against intracellular Campylobacter-like organisms.

Intestine designation	Age of pig	Farm origin of pig	Histological lesion	Immunofluorescence detected of intracellular organisms ^a
284/86	5 mo.	Dalrymple	PHE	+++
761/86	9 w.	Aberdeen	PIA	+++
1268/84	9 mo.	Bush	PHE	+++
153/85	10 w.	IAPGR	PIA	+++
428/81	5 mo.	IAPGR	PHE	+++
763/86	10 w.	Aberdeen	NE	++
42/78	14 w.	BG	None	-
Pig 1	17 d.	Chapter 2 Compton	PE	+++
Pig 2	27 d.	Chapter 2 Compton	PE	+++

PHE proliferative haemorrhagic enteropathy

PIA porcine intestinal adenomatosis

NE necrotic enteritis

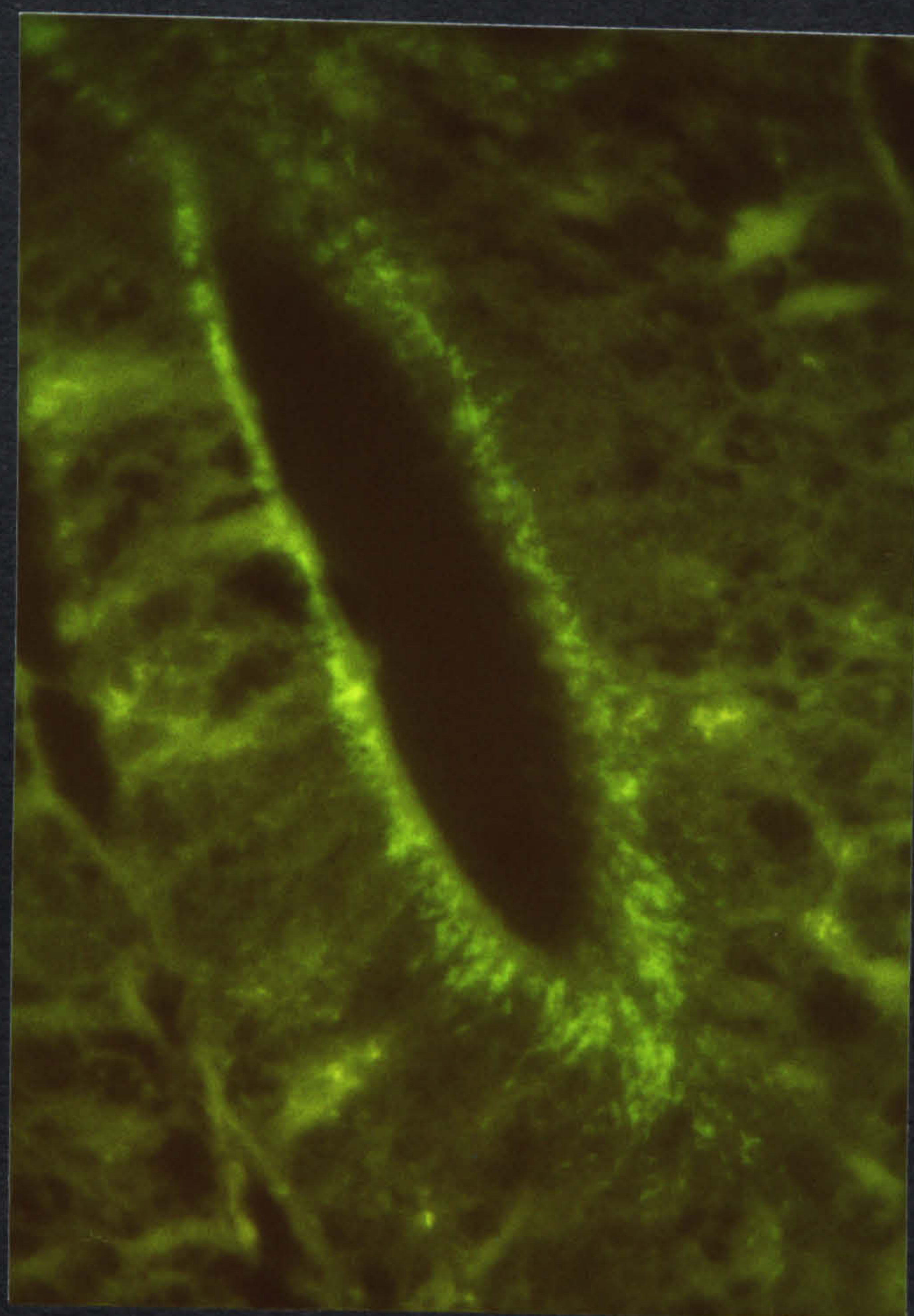
PE proliferative enteritis

a ++, +++ brightly fluorescing curved bacilli within enterocytes. - No fluorescence detected. Further description and figures in text.

FIGURE 26.

Photomicrograph of unstained section of ileum from a pig naturally affected by porcine intestinal adenomatosis (pig 153/85, see Table 16). Indirect immunofluorescence assay, with monoclonal antibody IG4 in neat supernatant fluid as the first antibody, and fluorescein-conjugated sheep anti-mouse immunoglobulin as the second antibody. Brightly fluorescing curved bacilli evident within the apical cytoplasm of enterocytes.

x 600.



proliferative enteritis had brightly fluorescing curved bacilli within the cytoplasm of enterocytes lining the crypts of proliferative mucosa and fluorescing bodies within mucosal mononuclear cells. This included hamsters 57/80 and 58/80, however hamster 56/80 (killed 7 days after infection) had brightly fluorescing curved bacilli within enterocytes in 10 per cent of the crypts in ileal sections. There was no histological evidence of enterocyte or crypt proliferation, nor of fluorescent material in the crypt lumen or in mononuclear cells in this hamster.

Sections of control pigs and hamsters showed no fluorescence.

Immunogold Staining of Intestinal Sections

The sections that were deosmicated, with or without trypsinising produced best results. Sections treated with monoclonal antibody and colloidal gold-protein A showed gold particles located along the middle of the outer membrane coats of Campylobacter-like organisms located deep within the cytoplasm of enterocytes within proliferative mucosa (Fig 27). Organisms of similar morphology located within adjacent crypt lumina and just within the apical cytoplasm of affected enterocytes, showed occasional gold particles within their structure. Only occasional particles were evident elsewhere, and within sections not treated with monoclonal antibody.

Indirect Immunofluorescence Assay of Faeces

The clinically affected pigs had numerous brightly fluorescing curved bacilli in faecal smears (Fig 28), and were used as positive controls for other examinations. Pigs 1 and 2 (Chapter 2) and two hamsters affected with proliferative enteritis, 21 days after infection (group 13, Chapter 3) had few to moderate numbers of brightly fluorescing curved bacilli in faecal smears. No specific

FIGURE 27.

Electron micrograph of ultra-thin section of proximal colon of pig 1 (10 days after infection), treated in an immunogold assay. Monoclonal antibody IG4 in supernatant fluid was the first antibody, and protein A-colloidal gold particles were the second stage. Fifteen nm particles evident in the outer membrane of the intracellular Campylobacter-like organisms deep within the cytoplasm of crypt enterocytes.

Uranyl acetate/lead citrate stain, x 77,000.

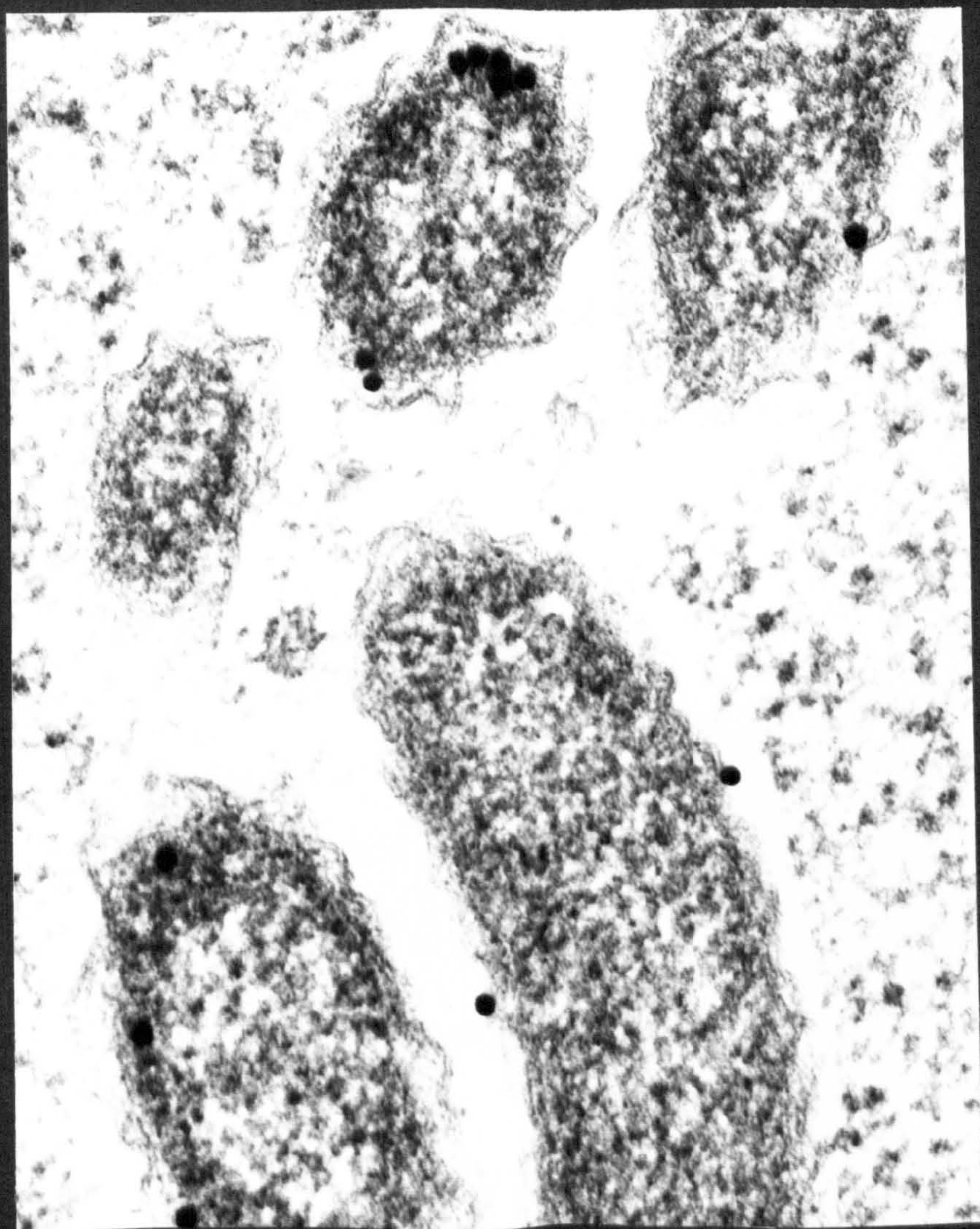
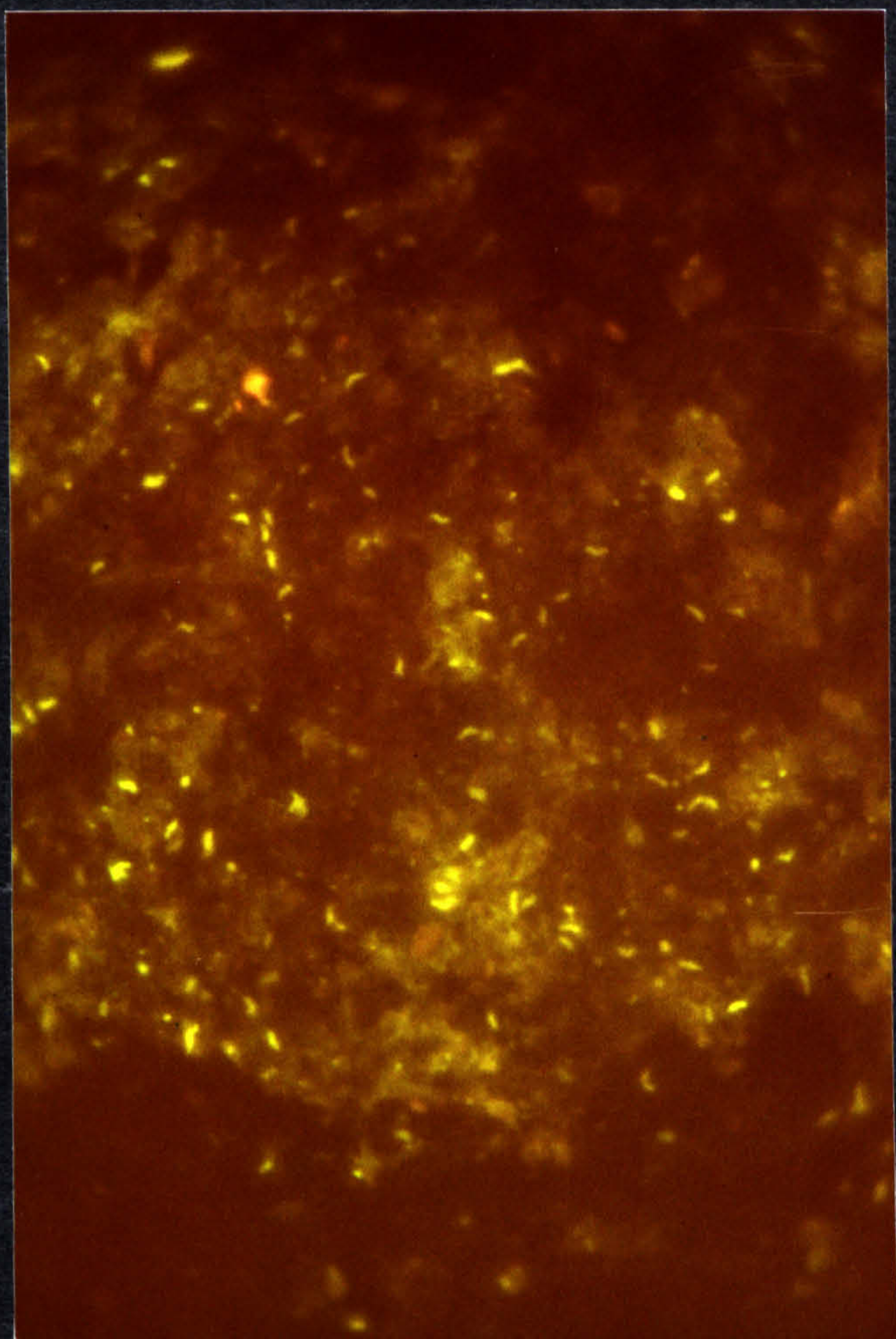


FIGURE 28.

Photomicrograph of a faecal smear from a pig 380/87, naturally affected by proliferative haemorrhagic enteropathy. Indirect immunofluorescence assay with monoclonal antibody 4F5 in neat supernatant fluid as the first antibody, and fluorescein-conjugated sheep anti-mouse immunoglobulin as the second antibody. Numerous fluorescing curved bacilli evident.

x 600.



fluorescence was detected in any other sample.

C.coli was cultured regularly (n = 90) from the faeces of 10 of the IAPGR pigs. Four hippurate-positive C.jejuni strains were cultured from one to two pigs between 5 and 12 weeks old, but not all isolates were fully identified. C.hyointestinalis was cultured from one to two pigs at each collection between 5 and 12 weeks and between 16 and 24 weeks old, for a total of 9 isolations. No clinical abnormalities were noted in these pigs.

Discussion

Campylobacter-like organisms within enterocytes in proliferative enteritis have an outer membrane component that is apparently specific for these organisms, and the organism within lesions in pigs and hamsters contain an identical antigen. Thus proliferative enteritis is associated with a Campylobacter-like organism apparently markedly different to known Campylobacter sp. The various forms of proliferative enteropathy in pigs, namely, necrotic enteritis, proliferative haemorrhagic enteropathy and intestinal adenomatosis, all contain intracellular Campylobacter-like organisms carrying a common antigen. Thus if Campylobacter-like bacteria are the cause of proliferative intestinal lesions, a common or closely related organism is involved and these bacteria are distinct from those Campylobacter spp currently recognized.

A further significant result is the demonstration of organisms specific for proliferative enteritis in the faeces of live animals, later confirmed as having proliferative enteritis at autopsy. This offers a great advantage in clinical diagnosis of the disease as serology or morphological examination of faeces have proved inadequate (Rowland and Lawson, 1986).

The immunofluorescence assays suggesting that the intracellular Campylobacter-like organisms are a single, uncultured bacterium affecting pigs and hamsters, supports the cross-species transmission reported in Chapter 3. Furthermore, the specific reaction of the antibodies with intracellular organisms, supports the immunofluorescence assays in Chapter 2, which identified some extracellular organisms with antisera to cultured Campylobacter sp, but no intracellular organisms.

In a separate study, monoclonal antibodies directed against C.jejuni/coli only detected extracellular organisms in lesions of proliferative enteritis in hamsters, whereas another antibody which showed general reactivity with several Campylobacter spp reacted with both the intracellular organisms, and some curved bacilli within crypt lumina (Stills et al, 1987). It is possible that the latter antibody detected a different Campylobacter or even a different bacterial genus within affected enterocytes to the one(s) in the lumina. Even so, these authors also suggested that the intracellular organisms were an as yet uncultured Campylobacter sp. Previous studies which reacted polyclonal rabbit antisera to cultured Campylobacter sp, with sections of lesions, reported staining of intracellular organisms in some instances (Chang et al, 1984a). However, studies reported in Chapter 4 and elsewhere (Lawson et al, 1985) established that some rabbit sera can react non-specifically with the intracellular organisms. The specific nature of monoclonal antibodies makes possible more confident conclusions.

The immunogold staining of intracellular organisms suggested that the monoclonal antibodies used, were directed against an outer membrane component. The identity of this component, presumably identical to the 25K to 27K one identified in immunoblotting, was not clear. Previous

immunogold studies of Campylobacter sp, have only identified flagellar components of 60K (Newell, 1986a). Immunogold staining of other bacteria has established that it is a useful technique for identifying the site of antigens (Beesley et al, 1982). The pattern of staining in the sections examined suggests that the specific component may be concentrated in organisms well below the enterocyte surface. The failure to recognize similar components on bacteria of similar morphology in associated crypt lumina suggests that either these bacteria are different, or that they undergo some antigenic change on entry into the cytoplasm of the enterocyte, or both.

Examination of faeces for organisms specific to proliferative enteritis proved useful in pigs clinically affected with proliferative haemorrhagic enteropathy. The apparent excretion of these organisms in these pigs suggests that there had been lysis of affected enterocytes, with release of the intracellular organisms into the bowel contents. This is consistent with the pathology of this form of proliferative enteritis (Rowland and Lawson, 1986). However the sensitivity of immunofluorescence examination of faeces for Campylobacter sp is likely to be low, with only approximately 40 per cent to 60 per cent of patients excreting C.jejuni/coli being detected in separate reports (Price et al, 1984; Hodge et al, 1986), corresponding to a detection limit of one million organisms per millilitre of faeces. The prospective examination of the faeces of IAPGR pigs did not reveal any significant findings, other than the culture of Campylobacter spp from healthy pigs. This suggests that the intracellular organisms associated with proliferative enteropathy are not ubiquitous or numerous in the lumen of the intestines of pigs. The isolation of C.hyointestinalis from normal pigs has not been reported previously. Previous assertions that it is closely associated with the lesions of

proliferative enteropathies (Gebhart et al, 1983) must therefore be examined cautiously.

CHAPTER 8

ISOELECTRIC FOCUSING AND IMMUNOBLOTTING OF
CAMPYLOBACTER SPP AND CAMPYLOBACTER-LIKE ORGANISMS

Introduction

Work described in previous chapters indicated that intracellular Campylobacter-like organisms in proliferative enteritis may contain a specific membrane component of 25K to 27K. However gel electrophoresis and immunoblotting (Chapter 5) could have altered this or other active components by chemical or heat denaturation. One method of avoiding such effects is to separate the components by isoelectric focusing.

Isoelectric focusing (IEF) separates sample proteins on the basis of their net charge, by placing them in a gel containing carrier ampholytes, which form a defined pH gradient across the gel, when a current is applied. The stronger the current (field strength), the greater is the resolution. The proteins migrate and accumulate at their particular isoelectric points (pI). Recent developments have made isoelectric focusing a relatively standard technique (Allen, 1980; Radola, 1980).

Two-dimensional gel electrophoresis, a combination of isoelectric focusing and vertical gel electrophoresis, was used to examine outer membrane components of some Campylobacter sp (Dunn et al, 1987). Several components were recognized within the flagellin component of the Campylobacter sp, which had previously been identified as a single component of 60K by gel electrophoresis (Logan and Trust, 1982).

The intention was to examine the specificity of the immune reaction to the 25K to 27K component in the absence of heat and reduction. Also, an examination was made for other possible reactions in sonicates of Campylobacter-like organisms and Campylobacter spp under such conditions, to the antibodies against Campylobacter-like organisms.

Materials and Methods

Antigen Preparation

Eight of the Campylobacter spp strains described in Chapter 4 and 5 (see Table 7), viz: C.mucosalis 1248/72, 124/73 B4, C.hyointestinalis 124/73A4, 9AL3, C.jejuni 1268/84J, 664/83, C.coli 9BF2, 9AF3a, were cultured on blood agar for 48 hours and collected into distilled water. A further strain, C.coli NCTC11353 was also prepared. After being washed twice in distilled water, each strain was suspended in distilled water to a density approximating Brown's opacity number 2, as measured by a nephelometer. Also, Campylobacter-like organisms were partly purified from mucosae 284/86 and 761/86 (see Table 8) by the method of mucosal homogenization and filtration described previously (see Appendix 5) using PBS as diluent. These organisms were further purified by passage through separate wheat-germ agglutinin-agarose columns (see Chapter 5), in PBS supplemented with calcium and magnesium ions (see Appendix 1). The organisms in the unbound fraction from each column were washed twice in distilled water and a standard suspension made as described above.

Each preparation was disrupted by treatment with ultrasonic waves in four 30 second bursts, as described previously. The sonicated suspensions were centrifuged (6,000g, 5 min.), and the supernatant fluid samples collected and stored in small aliquots at -20°C.

Preparation of Gel Slabs

Fifteen millilitres of an IEF stock acrylamide solution (see Appendix 2) was combined with 4ml of glycerol, 9ml of distilled, deionized water and 2ml of a proprietary mixture of carrier ampholytes pH4 to 8 (Pharmalyte, Pharmacia, Sweden), and degassed under vacuum for 10 minutes. Polymerization agents were added, 0.025ml of TEMED

(N,N,N,N-tetramethylethylenediamine), 0.3ml of 2.28 per cent w/v ammonium persulphate, and the mixture was poured into a proprietary capillary gel mould (Pharmacia, Sweden), to form a 1mm thick gel.

A polyester film, commercially silanized such that its surface was hydrophilic (Gelbond PAG, Pharmacia, Sweden), had previously been rolled onto the uppermost glass plate of the gel mould to allow attachment and support of the gel during polymerization. The set gel was removed with the film gel support and placed onto the focusing apparatus.

Isoelectric Focusing

Focusing was done on a flat-bed apparatus, with a Teflon-coated aluminium top (FBE-3,000, Pharmacia, Sweden). A cooling solution of 4°C was circulated through the apparatus from a separate thermostatic circulator (2219 MultitempII, LKB Instruments, U.K.). Two millilitres of kerosene was spread between the top and the gel support to improve heat exchange during focusing.

At both ends of the gel, 7mm wide electrode strips of thick cellulose paper (Pharmacia, Sweden) were placed along the length of the gel, 100mm apart. The strips had been soaked in 1.0N sodium hydroxide (catholyte) or 1.0M phosphoric acid (anolyte). Platinum ribbon electrodes were laid on the strips and covered with a glass plate (to prevent dehydration). Pre-electrophoresis was carried out at 3,000v, 150mA, 30W for a total of 500 volt hours. The electrode strips were replaced with fresh ones, and 0.010ml of each antigen sample placed directly on the surface of the gel, aided by a sample applicator strip. After re-connection, electrophoresis was conducted at the above settings, for 4,000 volt hours. Gels were fixed and stained by the Morrissey silver stain (see Appendix 3). No direct assessment was made

of the pH gradient; previous reports suggested it was likely to be linear (Radola, 1980; Dunn et al, 1987), and isoelectric markers (Sigma Chemical Co., U.K.) included in each run, indicated this also.

Immunoblotting

Further gels and samples were run as described above without staining. The gel was then separated from the polyester film, with a scalpel and cutting wire, and the electrophoresed components in the gel transferred to nitrocellulose paper sheets, by the electroblotting method described in Chapter 5. Separate sheets were then reacted with rabbit antiserum to Campylobacter-like organisms 1269/76, or hybridoma supernatant fluid IG4, using identical blocking, washing and colour development steps to those outlined in Chapters 5 and 6.

Results

The focused protein profiles of sonicated whole cell antigens of selected Campylobacter spp, as well as the products of mucosae 284/86, and 761/86 Campylobacter-like organisms after passage through separate wheat-germ agglutinin-agarose columns are shown in Figure 29. The profiles of the Campylobacter-like organisms show prominent proteins between pI 6 and 7, with much material still adjacent to the sample application sites at pI 4.5, and two prominent proteins around pI 5.0, particularly in the 284/86 preparation.

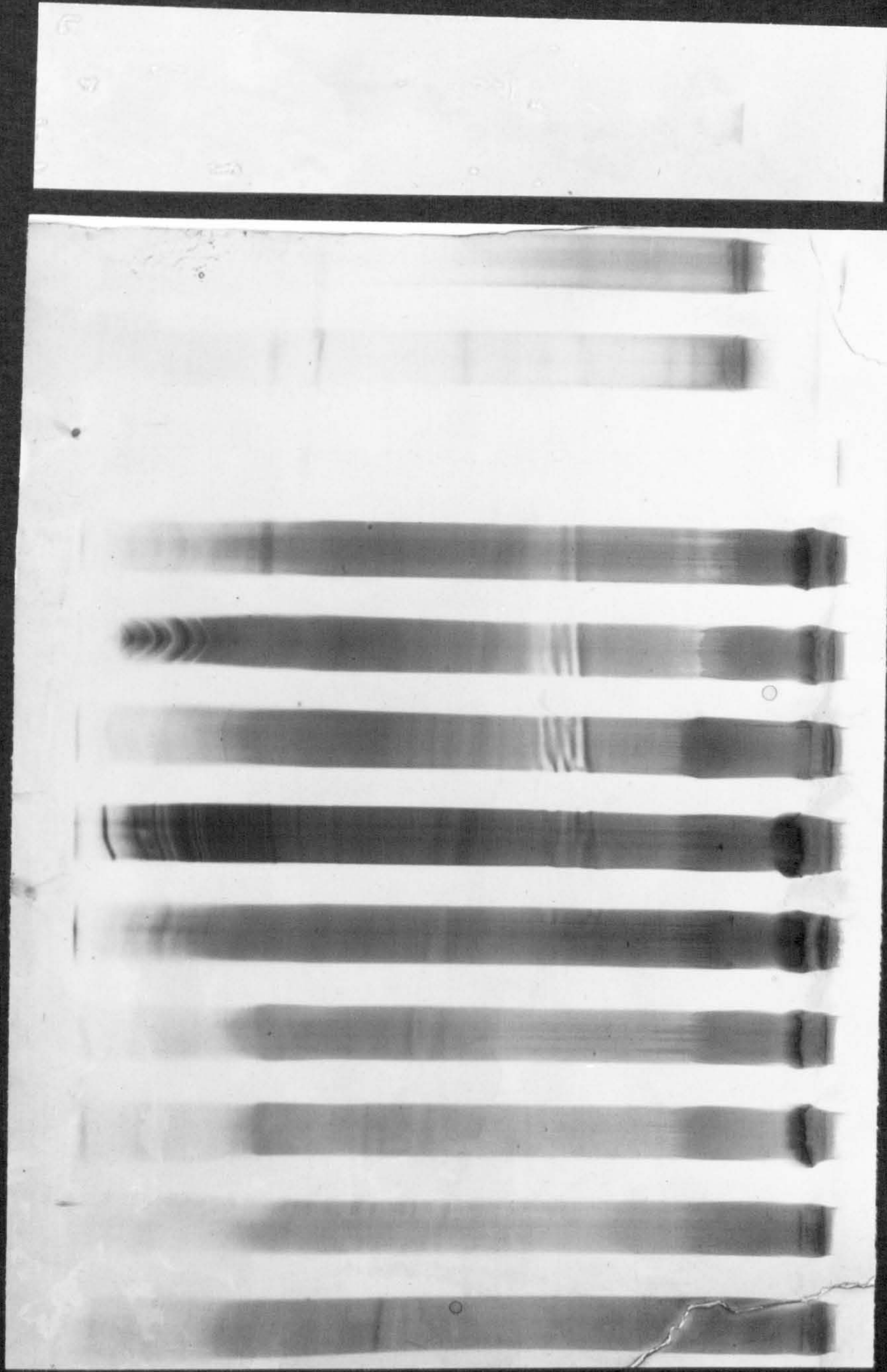
The profiles of C.mucosalis strains show a major protein at pI 6.5, particularly in 1248/72. The profiles of C.hyointestinalis, C.jejuni and C.coli strains show two proteins around pI 6.0, at slightly differing sites. In all the Campylobacter sp, proteins are evident between pI 7 and 8.

Antibodies in the antiserum to Campylobacter-like organism 1269/76 and in supernatant fluid IG4 only recognized the pI 4.5 components of

FIGURE 29.

Isoelectric focusing gel of preparations of Campylobacter-like organisms. Gel stained with silver method of Morrissey (1981). Lanes: 1, sonicated preparation of C.mucosalis 1248/72; 2, sonicated preparation of C.mucosalis 124/73B4; 3, sonicated preparation of C.hyointestinalis 124/73A4; 4, sonicated preparation of C.hyointestinalis 9AL3; 5, sonicated preparation of C.jejuni 1268/84J; 6, sonicated preparation of C.jejuni 664/83; 7, sonicated preparation of C.coli 9BF2; 8, sonicated preparation of C.coli 9AF3a; 9, sonicated preparation of C.coli NCTC 11353; 10, isoelectric marker, pI 4.0; 11, sonicated preparation of Campylobacter-like organisms 284/86; 12, sonicated preparation of Campylobacter-like organisms 761/86.

Immunoblot analysis. Lane 13; monoclonal antibodies in supernatant fluid IG4, reacted against Campylobacter-like organisms 284/86.



1 2 3 4 5 6 7 8 9 10 11 12 13

organisms from mucosae 284/86 (see Figure 29) and 761/86. These antibodies did not recognize components from any of the Campylobacter sp tested.

Discussion

Isoelectric focusing results suggested the existence of a specific component of pI 4.5 within Campylobacter-like organisms in lesions of proliferative enteritis, with an antigenic site identical to that of the 25K to 27K component detected in immunoblotting of separations in reducing gels (Chapters 5 and 6). Therefore it is likely that the components detected by the two methods represent the same structural component. Also, as isoelectric focusing is a non-denaturing method, it suggests that there is a valid, single component and that the previous reducing gels did not cause denaturation of other significant components.

As isoelectric focusing is a remarkably sensitive technique for separation of proteins, and the antibodies used only detected reactive components in the intracellular organism preparations, this further suggests that these organisms are markedly different from known Campylobacter sp.

CHAPTER 9

ANALYSIS OF DNA WITHIN CAMPYLOBACTER SPP AND
CAMPYLOBACTER-LIKE ORGANISMS ASSOCIATED WITH
PROLIFERATIVE ENTEROPATHY

Introduction

Analysis of chromosomal deoxyribonucleic acid (DNA) is now recognized as the major taxonomic criterion for differentiation of bacterial species, including Campylobacter sp (Veron and Chatelain, 1973; Smibert, 1984). However complete DNA hybridization techniques for this purpose are expensive and tedious, so are only conducted at few, specialised laboratories.

However two simpler techniques have recently allowed similar taxonomic accuracy, without the need for specialised facilities. First, preparation of a radio-labelled probe to a portion of bacterial DNA, enables it to be reacted with other strains (Moseley et al, 1980). Second, digestion of the bacterial DNA by an appropriate restriction endonuclease gives a specific mixture of DNA fractions when visualized in agarose gels (Bradbury et al, 1985). The second technique was investigated in this study to compare the DNA in known Campylobacter sp to that in the intracellular Campylobacter-like organisms in proliferative enteritis.

A survey of the plasmid DNA content of various Campylobacter sp was also conducted, as plasmids can be co-purified with chromosomal DNA, confusing the restriction endonuclease pattern. Furthermore, plasmids are a significant feature of many enteropathogens, including C.jejuni/coli (Bradbury and Munroe, 1985), but no data was available for other Campylobacter sp.

Materials and Methods

Chromosomal DNA

Bacterial preparations

Six of the Campylobacter sp strains described in Chapters 4 and 5 (see Table 7), viz. C.mucosalis 1248/72, 124/73B4, C.hyointestinalis

124/73A4, 9AL3, C.jejuni 1268/84J and C.coli 9BF2, were cultured on blood agar for 48 hours and collected into 0.9 per cent w/v sodium chloride, in 1.5ml Eppendorf tubes. Campylobacter-like organisms were partly purified from mucosae affected by proliferative enteritis, 284/86 and 1269/76 by homogenization and filtration as described in Appendix 5, with PBS as diluent. Organisms were further purified by passage through a wheat-germ agglutinin agarose column (see Chapter 5). Purified organisms were finally collected into 0.9 per cent w/v sodium chloride, and smears of these preparations stained by modified acid-fast method indicated that Campylobacter-like organisms were the major (>95 per cent) component. Cultures of these preparations were negative for Campylobacter sp.

Tissue preparations

As a control for the Campylobacter-like organism preparations, liver, thymus and two ilea (all designated 167/88) were collected at an abattoir from freshly killed pigs (4 months old) and transported frozen back to the laboratory. The mucosa of each ileum was scraped into separate vials.

Chromosomal DNA preparation

The method used was modified from that of Bradbury et al (1984). Bacterial preparations were centrifuged (3,800g, 3 minutes) and each pellet mixed with 0.3ml of DNA lysis buffer (see Appendix 1) for 10 minutes at 37°C. 0.15ml of 10 per cent w/v sodium lauryl sulphate was added, and each tube mixed for 10 minutes at 20°C. 0.45ml of a fresh, 1:1 phenol-chloroform solution (see Appendix 1) was added, and an emulsion created by vigorous mixing. After centrifugation of precipitated protein (6,400g, 5 minutes), the upper aqueous phase was collected into a fresh tube. An equal volume of the phenol-chloroform

was added, and the precipitation/extraction process repeated. Ribonuclease solution (see Appendix 1) was added to the aqueous phase, to a final concentration of 0.050mg ml^{-1} and the tubes incubated at 37°C for 30 minutes. An equal volume of chloroform was added, vortexed and centrifuged as above. The upper aqueous phase was carefully collected into a fresh tube, and two volumes of ice-cold 95 per cent v/v ethanol was added, vortexed then the tubes chilled to -70°C for one hour. After centrifugation ($12,000g$, 10 minutes) the supernatant fluid was completely removed and 0.250ml of DNA wash buffer (see Appendix 1) added to dissolve the pellet. After storage overnight at 4°C , the ethanol precipitation was repeated, and the pellet of DNA dissolved in warm distilled water. DNA was stored at -20°C , after spectrophotometric determination of the DNA quantity and purity (Maniatis et al, 1982).

For the preparation of chromosomal DNA from tissue samples, one gram of each sample was homogenized in a blender (M.S.E. Instruments, U.K.), then mixed with 40ml of one per cent w/v sodium lauryl sulphate in Tris-EDTA buffer (see Appendix 1), containing 0.1mg ml^{-1} proteinase K (Sigma Chemical Co., U.K.), for two hours at 65°C . An equal volume of phenol-chloroform was added to the lysed cells, and subsequent precipitation, extraction and ribonuclease addition steps performed as described above.

Restriction Endonuclease Digestion of DNA

Seven restriction endonucleases were purchased (Pharmacia Fine Chemicals, Sweden) and appropriate assay buffers prepared (see Table 17) according to the manufacturer's instructions. Approximately 0.05ml of DNA solution of each Campylobacter sp (approximately $5\mu\text{g}$ DNA) was diluted in 0.2ml of the relevant assay buffer containing 50 units of

TABLE 17: Restriction endonucleases

Restriction endonuclease	Origin	Cleavage site sequence	Tris-HCl ^a	pH	Assay buffer Sodium ^a chloride	Magnesium ^a chloride	mercapto-a ethanol	BSA ^b
AvaII	<u>Anabaena variabilis</u>	G/G(A,T)CC	10	8.0	60	10	1	0.1
BglII	<u>Bacillus globigii</u>	A/GATCT	20 ^c	9.5	200	10	7	0.1
EcoRI	<u>Escherichia coli</u>	G/AATTC	10	7.5	100	10	1	0.1
HhaI	<u>Haemophilus haemolyticus</u>	GCG/C	10	8.0	50	10	1	0.1
HindIII	<u>Haemophilus influenzae</u>	A/AGCTT	10	7.5	60	10	1	0.1
KpnI	<u>Klebsiella pneumoniae</u>	GGTAC/C	10	7.5	10	10	10	0.1
PvuII	<u>Proteus vulgaris</u>	CAG/CTG	10	7.5	60	10	10	0.1

a Values expressed as mM in assay buffer.

b BSA - bovine serum albumin treated for removal of DNase activity (Sigma). Values expressed in mg ml⁻¹.

c Glycine - hydroxide buffer instead of Tris-HCl.

each enzyme, and incubated for 16 hours at 37°C. Enzymes giving suitably resolved DNA restriction fragments were incubated in a similar manner with DNA solutions of each preparation of Campylobacter-like organisms and of each tissue.

Agarose gel electrophoresis

Native (undigested) DNA preparations and digested DNA samples were mixed 5:1 with concentrated DNA sample buffer (see Appendix 1). Two grams of agarose (Agarose NA, Pharmacia Fine Chemicals, Sweden) was dissolved by boiling in 200ml of Tris-acetate-EDTA buffer (see Appendix 1), cooled to 70°C, then poured into a horizontal slab gel apparatus (Pharmacia GNA - 200), giving a 1 per cent gel 0.5 x 20 x 20cm. The gel was immersed ("submarine" mode) in the Tris-acetate-EDTA buffer with passive circulation of the buffer between each end of the gel. Samples of approximately 2µg of DNA were added to wells at the cathodic end of the gel, electrophoresed for 16 hours at 30v, stained for one hour in 0.001mg ml⁻¹ ethidium bromide in water and destained for 10 minutes in distilled water. DNA fragments were visualized by illumination with ultra-violet light at wavelength 302nm (UV Transilluminator TM36, U.V.P. Inc., U.S.A.) and photographed by a filtered (Wratten Orange filter no. 23A) Polaroid camera. DNA samples were digested and analysed at least twice, where sufficient endonuclease was available.

Plasmid DNA

Bacteria

An expanded number of strains were examined for plasmids:
C.mucosalis 1248/72, 124/73B4, 1494/72, 900/74, 722/75, 140/76, 1370/76, 1371/76, 1405/76, 921/77, 108/80, 691/82 CSM, 716/86;
C.hyointestinalis 124/73A4, 632/74A2-VC, 691/82 CHI; C.jejuni 1268/84J;
C.coli 1268/84K. All these strains (n = 18) were cultured from the

intestines of natural cases of proliferative enteritis in pigs (n = 15) and had been deposited in the culture collection of the Royal (Dick) School of Veterinary Studies, Edinburgh. Other strains mainly isolated during the course of this study viz: C.hyointestinalis 9AL2, 9AL3; C.jejuni 664/83; C.coli 9BF2, 9AF3a, 60/87, see Chapters 2 and 3, were also included.

Each strain was cultured on blood agar for 48 hours and collected into Tris-EDTA buffer (see Appendix 1). Campylobacter-like organisms purified as for chromosomal DNA, were also included.

Plasmid DNA preparation and electrophoresis

Plasmid DNA was extracted by the rapid alkaline extraction procedure (Birnboim and Doly, 1979). Briefly, each pellet of bacteria was suspended in 0.2ml of DNA lysis buffer for 10 minutes at 20°C and 0.2ml alkaline-SDS added (see Appendix 1). After being on ice for 5 minutes, 0.15ml of concentrated acetate solution (see Appendix 1) was added. After mixing, the tube was left on ice for 5 minutes then centrifuged (12,000g, 3 minutes). 0.4ml of supernatant fluid was removed, the DNA in it was extracted with ethanol twice, and mixed with ribonuclease, in Tris-EDTA buffer (see Appendix 1).

Samples were mixed with DNA sample buffer (see Appendix 1) and electrophoresed in horizontal slab gels as described above, except that 0.7 per cent gels were used.

In all gels, DNA molecular weight (base pair no.) markers were included (Lambda DNA Hind III digest, Sigma Chemical Co., U.K.), for comparison.

Results

Chromosomal DNA

Restriction endonuclease digestion of Campylobacter sp with either AvaII or BglII gave suitably resolved DNA fragments between 2kb and 25kb. Digestion with EcoRI or Hind III gave numerous fragments, but resolution of these was poor on electrophoresis, despite repeated efforts. Attempted digestion with other endonucleases did not produce fragments.

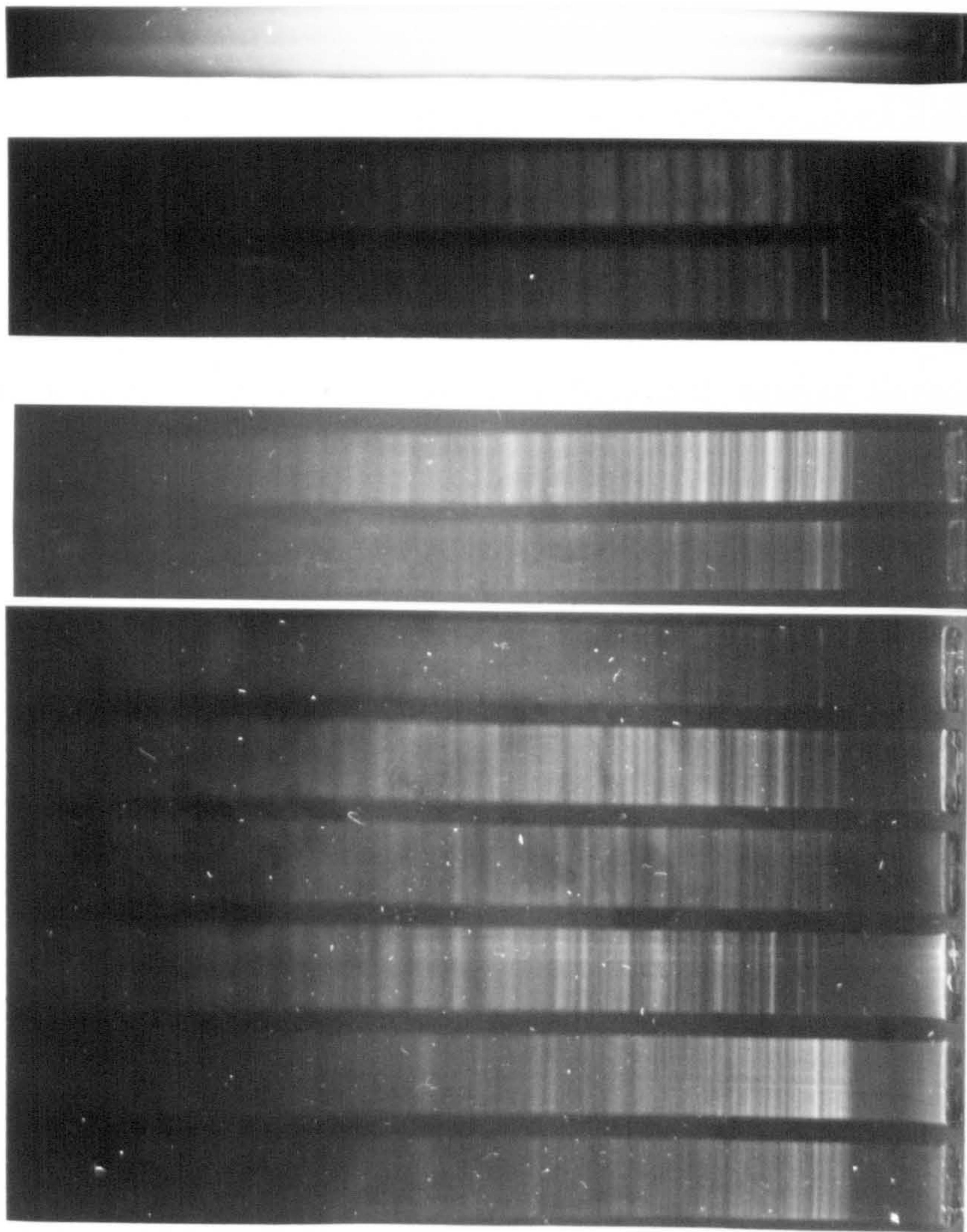
Digestion of DNA preparations of Campylobacter-like organisms from mucosae gave poorly resolved fragments, when AvaII was used. However, digestion with BglII produced clearly resolved fragments between 2kb and 25kb, which allowed comparison with each other and with those of Campylobacter sp. Repeated digestions gave similar results. Figure 30 illustrates that patterns obtained with BglII digestion of DNA from Campylobacter-like organisms obtained from mucosae 1269/76 and 284/86 appeared similar (lanes 9 and 10). Approximately 90 per cent of fragments ($n > 25$) were in identical positions, for example, the fragment at 7.2kb.

Figure 30 also illustrates that patterns obtained with BglII digestion of Campylobacter sp were dissimilar to those of Campylobacter-like organisms, and that each Campylobacter sp had a characteristic distinct pattern. Digestion of DNA from tissue samples with BglII produced a diffuse smear of fragmented DNA bands between 0.5 and 19kb, with no recognizable "ladder" effect, see Figure 30.

Complete digestion was indicated by the lack of high molecular weight fragments in any lane. The 260nm to 280nm absorption ratios of the native DNA preparations were all 1.80 ± 0.05 . Native DNA samples of each preparation run on gels, invariably formed a single high molecular weight band, except for C.coli 9BF2, which contained bands at

FIGURE 30.

Agarose gel electrophoresis of preparations of DNA from Campylobacter spp, purified Campylobacter-like organisms and porcine tissue digested with BglII. Gels stained with ethidium bromide, and photographed under ultra-violet illumination. Lanes: 1, C.mucosalis 1248/72; 2, C.mucosalis 124/73 B4; 3, C.hyointestinalis 124/73A4; 4, C.hyointestinalis 9AL3; 5, Campylobacter-like organisms 1269/76; 6, Campylobacter-like organisms 284/86; 7, C.jejuni 1268/84J; 8, C.coli 9BF2. Separate gel lanes: 9, Campylobacter-like organisms 284/86; 10, Campylobacter-like organisms 1269/76; 11, porcine ileum 167/88. Molecular weights indicated, are expressed in Kilobase pairs (Kb).



2→
2.3→

4.4→

6.5→

9.4→

23→

1 2 3 4 5 6 7 8 9 10 II

3.0 and 5.6kb corresponding to plasmid DNA (see below).

Plasmid DNA

Plasmid DNA was observed in 5 strains, viz: C.jejuni 664/83 (35, 80kb), C.coli 9BF2 (3.0, 5.4kb), C.coli 60/87 (28, 35, 100kb), C.hyointestinalis 9AL2 (45, 60kb), C.hyointestinalis 9AL3 (45, 60kb), plasmid sizes in kilobase pairs are given in parentheses. Plasmid profiles were consistently reproducible. The profile for C.hyointestinalis 9AL2 and 9AL3 appeared to be identical, but other profiles were different to this and each other. No strains isolated or organisms purified from naturally occurring proliferative enteritis contained detectable plasmids, although few C.jejuni/coli strains were examined.

Discussion

Restriction endonuclease digestion of the DNA of each Campylobacter sp and the Campylobacter-like organisms associated with proliferative enteritis produced fragment patterns which differed strikingly from each other. The genome of the Campylobacter-like organisms within enterocytes in proliferative enteritis therefore may be different to that of known Campylobacter spp associated with the disease. This suggests that the differences in antigenic structures between these bacteria noted in previous Chapters, may be due to genetic differences. Only a limited number of strains were examined. The possibility that the preparations of Campylobacter-like organisms contained DNA from host tissue or other sources was investigated by the incorporation of tissue DNA preparations into the study. The tissue DNA digests had a large number of fragments, including many not present in digests of DNA from the Campylobacter-like organisms, i.e. where "gaps" appeared in the latter. Therefore it is likely that the

Campylobacter-like organism preparations did not contain a significant amount of tissue DNA. However, as no previous studies have incorporated bacteria derived from tissue sources as part of restriction endonuclease analysis, it is not possible to make definitive statements about the genetic differences between the Campylobacter-like organisms and Campylobacter spp, based on comparable work. Even so, the differences in restriction endonuclease fragment patterns would tend to suggest that there are genetic differences.

The validity of the interpretation that restriction endonuclease fragment patterns do indicate significant genetic similarities or otherwise for cultivable bacteria, has been substantiated by several studies linking this analysis, with cultural and/or DNA hybridization studies (Marshall et al, 1981; Collins and Ross, 1984; Bradbury et al, 1985). These indicate that organisms from within one species show markedly different fragment patterns to the patterns of organisms from other species of the same genus. There can be less marked, but significant differences in the fragment patterns between strains within a species. Organisms from the same source or serogroup all tend to show an identical fragment pattern, but this pattern can differ slightly from that of organisms from other sources (Bradbury et al, 1984; De Lisle et al, 1987).

The endonucleases used in previous studies have varied, and Hind III, BstEII and XhoI have been suggested as suitable for Campylobacter sp (Collins and Ross, 1984; Bradbury et al, 1984), Hind III because Campylobacter sp have a low content of guanine-cytosine and its cleavage site (see Table 17) does not involve these bases. However, BglII produced acceptable digestion and clear resolution of fragments of suitable size in this study, even though its cleavage site does involve guanine. Furthermore, two separate C.mucosalis strains, of

different source and serotype (I and III, see Lawson et al, 1977) gave nearly identical patterns. The two preparations of Campylobacter-like organisms also gave nearly identical patterns; even though they were both from lesions of proliferative haemorrhagic enteropathy, they were separated by a ten year collection difference and were from different sources. However, there were some differences in the two C.hyointestinalis strain patterns. A recent preliminary study found that C.hyointestinalis strains had four slightly different BglII digestion patterns, and that these patterns were related to serogroup reactions (Wesley and Bryner, 1987). It may be that the C.hyointestinalis differences seen in this study are due to these strain differences. The limited number of strains of each species examined in this study does not allow firm conclusions. Similarly, the failure to examine DNA from Campylobacter-like organisms extracted from intestinal adenomatosis material, due to technical difficulties and the difficulty in removing culturable Campylobacter sp from such material, means that the conclusions on the genome differences in relationship to the identity of the intracellular organisms must be limited.

However, if the organisms from proliferative haemorrhagic enteropathy mucosae 1269/76 and 284/86 have indeed a different genome to known Campylobacter sp, then the clear identification of these organisms with specific monoclonal antibodies (Chapters 6 and 7) which also reacted with organisms in other types of proliferative lesions, indicates that any genomic differences may extend to intracellular organisms in other forms of the disease. Thus it is possible to speculate that the intracellular Campylobacter-like organism in the lesions of the proliferative enteropathies is a separate, novel bacterium worthy of a new name. Such a conclusion would need to be explored by further comprehensive study, but there are clearly

considerable practical and technical difficulties to be overcome before such a step is possible. Further discussion of these taxonomic issues is given in Chapter 10.

The relationship of the genome of Campylobacter-like organisms in this study, to the expression of the 25 to 27K component detected in their outer membranes (Chapter 5) is not clear. The gene encoding for this component may only be present in the Campylobacter-like organism genome. Alternatively, it could be present in other Campylobacter sp(p) where its expression is suppressed or induced depending on the bacteria's environment. Significant antigen alterations have been detected in Campylobacter spp and other Gram-negative bacteria during in vivo or in vitro manipulations and possibly reflect altered gene activity (Corbeil et al, 1973; Lawson et al, 1977; Lambden et al, 1979; Munn et al, 1982). However to answer this issue of the origin of the 25 to 27K component, a significantly more complex study is required. Portions of the DNA of the Campylobacter-like organisms could be spliced into phages, which could then be inserted into Escherichia coli, and the protein products produced by E.coli recombinants analysed with the monoclonal antibodies. A clone of any positive colony could provide the DNA producing the component, which could then be radiolabelled for use as a probe for hybridization studies. This "expression library" technique has proved useful for DNA analysis of other unculturable bacteria such as Treponema pallidum (Stamm et al, 1982).

It should be noted however, that the genes encoding for virulence factors of intracellular bacteria which are associated with the gastrointestinal tract are largely carried on plasmids (Formal et al, 1983). Plasmid-encoded outer membrane proteins are vital for the attachment, entry and pathogenic effects of Salmonella spp, Shigella

spp and Yersinia spp. In this study, plasmids were not detected in Campylobacter spp isolates from naturally occurring proliferative enteritis, but were seen in a high proportion of C.jejuni/coli isolates. In contrast to the enteropathogens mentioned above, no significant virulence factor has been ascribed to any plasmid from Campylobacter jejuni (Taylor et al, 1984, 1987). Therefore it is likely that chromosomal DNA analysis offers more scope for investigation.

CHAPTER 10
GENERAL DISCUSSION

The aim of this study was to characterise the intracellular Campylobacter-like organisms associated with the lesions of the porcine proliferative enteropathies. During the study, experimental results suggested several previously unrecognized features of these organisms. First, they are vital components of inocula for the successful transmission of the disease to gnotobiotic piglets, and similar inocula can induce lesions of proliferative enteritis in another host, the hamster. Second, they possess an outer membrane component of 25K to 27K molecular weight, pI 4.5. This component is apparently specific to the intracellular organisms, and was not found in normal pig intestine or in known Campylobacter species previously associated with the disease. Organisms possessing this component were the major intracellular bacterial species in all the various clinico-pathological forms of proliferative enteritis in both pigs and hamsters. This suggested that the intracellular Campylobacter-like organisms from these various sources are identical or very closely related. Third, the chromosomal DNA of the intracellular organisms is dissimilar to that of the known Campylobacter species, as assessed by restriction endonuclease analysis.

These features, and previously published evidence provide arguments for two possible conclusions about the identity of the intracellular Campylobacter-like organisms.

First, the intracellular organisms could be one of the known Campylobacter species previously associated with proliferative lesions, such as C.mucosalis, C.hyointestinalis or C.coli, with the provision that an antigenic change occurs in the bacteria's entry into affected enterocytes.

Arguments for this conclusion are that there is a clear cultural association between C.mucosalis and intestinal adenomatosis lesions,

and to a lesser extent C.hyointestinalis and various proliferative lesions in pigs (Lawson and Rowland, 1974; Gebhart et al, 1983). Also, either of these two species have been cultured from both the inocula and lesions in experimentally affected pigs, in successful transmission experiments (Roberts et al, 1977; Lomax et al, 1982b; Mapother et al, 1987b). Also, application of C.mucosalis to epithelial cells in tissue culture leads to intracellular entry and multiplication of the bacteria (Rajasekhar, 1981). Alteration of the antigens of bacteria, including Campylobacter species, in response to host or environmental factors is a well described phenomenon (Munn et al, 1982; Field et al, 1986; Blaser et al, 1987).

Arguments against this conclusion are that no bacterium in pure culture has proved capable of reproducing the lesions of proliferative enteritis, in pigs or hamsters. Neither C.mucosalis nor C.hyointestinalis have been associated with proliferative lesions in hamsters, despite the connection between these lesions and those in pigs, established in this study. Similarly, lesions of proliferative enteritis in pigs have been investigated where neither organism was cultured (Roberts et al, 1979). Experimental reproduction of proliferative enteritis in pigs has not been clearly associated with one or other cultured organism in the various published studies. Also, immunofluorescence assays involving rabbit antisera to these organisms, have not reliably demonstrated reactions with the intracellular organisms (Lawson et al, 1985).

The second possible conclusion is that the intracellular organisms could be a separate, new species of Campylobacter. The assertion that they are a Campylobacter sp is supported by morphological examination of affected tissue (Rowland and Lawson, 1974) and their reaction with antibodies having general reactivity with Campylobacter spp (Stills et

al, 1987)). However, neither of these studies was conclusive, and it is possible that the intracellular organisms are unrelated to Campylobacter sp. All known Campylobacter sp live in an extracellular, rather than an intracellular environment. Therefore if the intracellular organism of the proliferative enteropathies is, in fact, an obligate intracellular bacterium, then it should be assigned to a genus separate to the Campylobacter. Also, there was no evidence of flagella or flagellin components in this, or previous studies. All know Campylobacter sp have a prominent, polar flagellum, which is probably an evolutionary advantage in those species living in the intestinal lumen (McSweeney and Walker, 1986; Lee et al, 1987). Other bacteria known to be capable of growth in the cytoplasm of epithelial cells include Bacillus piliformis, Chlamydia sp and various Rickettsia spp. However, many Rickettsia spp require insect vectors for their life cycle, and most of these bacteria do not bear close morphological similarity to the intracellular organism of the proliferative enteropathies. At present, the organisms most closely resemble Campylobacter sp.

Arguments for their being a new species, are that successful experimental reproduction of the disease in pigs and hamsters has only occurred when inocula containing numerous intracellular organisms have been used. In this study, proliferative enteritis occurred following oral inoculation of gnotobiotic piglets with an inoculum containing numerous intracellular Campylobacter-like organisms, with few viable C.coli cultured. Rabbit antisera or monoclonal antibodies prepared against purified intracellular organisms reacted specifically with these organisms, with few or no cross-reactions to known Campylobacter sp. The electrophoresis of bacterial structural proteins, and chromosomal DNA restriction endonuclease fragments on gels, showed

different patterns between each Campylobacter sp and preparations of intracellular organisms.

Arguments against this second conclusion, are that the culture of C.mucosalis, C.hyointestinalis and C.jejuni/coli from the lesions becomes an unexplained phenomenon, of lesser significance, in contrast to their previous association. Also, no "new" Campylobacter sp has been recognized on culture of natural or experimental lesions of proliferative lesions.

Looking at the evidence provided by this study, the overall tenor of the results suggest that the second conclusion has more validity. If indeed the intracellular organisms are a single, new Campylobacter species, then a new name may be proposed, such as "C.intracellulare". If, as discussed above, the organisms are an obligate intracellular bacterium different to Campylobacter sp, then a new genus would have to be established. Verification of the validity of either proposal would require further DNA studies. The implied failure to culture the intracellular organisms, may have been due to technical faults, such as inappropriate atmosphere or media, or to its intracellular adaptation making it unsuitable for in vitro culture.

A further point to be addressed is whether the intracellular Campylobacter-like organisms are the sole factor in the pathogenesis of proliferative enteritis. There has been speculation that the initiation of the lesions may require host infection with an enteric virus such as rotavirus or enterovirus. These viruses have been isolated from proliferative lesions in pigs, and 0.22 μ m filtrates of proliferative mucosa can produce a non-specific enteritis in piglets (Mapother et al, 1987a; Joens et al, 1987).

However, the pathogenesis of experimental lesions in gnotobiotic piglets (Chapter 2) and in hamster studies, suggested that significant

entry of Campylobacter-like organisms into crypt enterocytes, and associated proliferation of such enterocytes had occurred by five to ten days after oral inoculation of infectious mucosal material. This suggests that the early lesions of proliferative enteritis develop in specific relationship to the presence of intracellular organisms, with a limited time for any proposed viral effect. Furthermore, known infectious mucosae showed complete loss of infectivity following exposure to tetracyclines, or filtration through 0.22 μ m or 0.45 μ m filters (Jacoby et al, 1975; Regina et al, 1980), suggesting that bacteria were the active ingredient. Also, addition of C.jejuni to proliferative enteritis mucosa (Chapter 3) did not cause any alteration in the infectivity or otherwise of the test inocula. This suggested that there was no viral or other agent present within proliferative mucosa, which needed a Campylobacter sp for expression of the disease.

Therefore it is likely that viruses are not a common primary factor in the pathogenesis of proliferative enteritis. Their presence may be incidental or even contributory to the lesions in some cases, however they are probably not essential to their development.

The future investigation of the proliferative enteropathies could include the following three areas. First, if the intracellular organisms are a new, uncultivated organism, then attempts at isolation could be made on cell culture systems. A possible method for such isolation, would be to homogenize a proliferative enteropathy mucosa, to release intracellular Campylobacter-like organisms, and incubate the suspension with a cell monolayer, to allow adaptation of the organisms to intracellular growth in the new cells. However, there would be numerous contaminating bacteria, including some Campylobacter sp in such an inoculum suspension, which could grow in the extracellular medium and cause death of the cell culture. Some antibiotics, such as

neomycin, are known to have little effect on the infectivity of proliferative enteritis mucosa (Regina et al, 1980), therefore these could be added to the medium and inoculum to aid the control of contaminating bacteria.

The choice of a suitable cultured cell line would be largely empirical. Porcine or hamster enterocyte cell lines are not available commercially, and are likely to be difficult to grow in vitro, from primary cultures. Rat, mouse and human enterocytes, as well as other epithelial cell lines (for example hepatocytes), are available commercially, but are of unknown usefulness. If, however a particular cell line, for example rat enterocyte, was established to be capable of allowing intracellular growth and multiplication of the Campylobacter-like organisms, under regular passaging, then a number of important experiments could be planned. Such experiments would include preparation of Campylobacter-like organisms free of other bacteria or porcine material, allowing study of specific antigens and DNA sequences. Also, development of the culture of the Campylobacter-like organisms on agar medium would be facilitated, and infected cell cultures could be used as an inoculum in animal experiments.

Second, if the intracellular organisms prove difficult to cultivate in vitro, then information on their antigenic and genetic structure could be obtained by use of recombinant DNA technology. The preparation of genetic material from messenger RNA extracts is likely to be too difficult due to insufficient available RNA. In such circumstances, recombinant genetic material can be prepared and characterised, by use of the expression library technique. This technique has been previously applied to Treponema pallidum and Mycobacterium tuberculosis (Stamm et al, 1982; Young et al, 1985). In the latter study, bacterial DNA digested by a restriction endonuclease

was separated in an agarose gel, and portions packaged into suitable lambda phages. Phages containing separate portions of DNA were cultured in individual Escherichia coli colonies and those colonies producing proteins recognised by relevant monoclonal antibodies, were selected for further antigenic and genetic study. Such an approach could be used for the Campylobacter-like organisms, as it has been established that suitable endonuclease and monoclonal antibody reagents are available, and the relevant phages and enzymes are available commercially. The separate portions of DNA prepared, could also be used in standard DNA-DNA hybridization assays, with DNA from other bacteria, such as Campylobacter sp.

Third, a further series of gnotobiotic piglet experiments could be planned. One candidate inoculum would be a purified preparation of intracellular Campylobacter-like organisms, free of other agents. The results of Chapter 2 suggest that such an inoculum may well reproduce proliferative enteritis. However, significant problems are likely in the production of a pure preparation from diseased intestine. The methods used in this study resulted in preparations containing some Campylobacter sp and other bacterial and porcine material. Lectin affinity chromatography produced cleaner preparations, but only small numbers of organisms. Purification of Campylobacter-like organisms could be achieved by use of the specific monoclonal antibodies produced in this study in immuno-affinity techniques. However these techniques are also only likely to produce small numbers of infective organisms. The viability of contaminating Campylobacter sp and other bacteria in a candidate inoculum could be reduced below detectable limits by addition of suitable antibiotics, such as neomycin (see above), or antisera capable of absorbing these bacteria. All of these purification techniques are likely to substantially reduce the infectivity of any

inoculum.

In conclusion, evidence has been presented for a novel bacterium, which is the major aetiological agent of the proliferative enteropathies. While little is yet known of this bacterium, the following is a description of the agent:

The cells are delicate, curved, gram negative and resist decolourization with 0.5 per cent acetic acid. The cells are 0.2 to 0.3 μ m in diameter and 1.5 to 4 μ m long, with rounded or tapered ends and no visible spores or flagella. The cells have an outer, tri-laminar wavy coat that is separated from the cell membrane and cytoplasm by an electron-luscent zone. Detected within the cytoplasm of enterocytes of pigs and hamsters with proliferative enteropathy.

APPENDICES

Appendix 1 - Buffers

Phosphate-buffered saline - 8.0g of sodium chloride, 0.2g of potassium dihydrogen phosphate, 2.9g of disodium hydrogen phosphate $12H_2O$, 0.2g of potassium chloride per litre of distilled water, pH7.4.

ELISA dilution buffer - phosphate-buffered saline, with 0.05% v/v Tween 20 and 0.1% w/v bovine serum albumin (Sigma).

Lectin buffer 1 - phosphate-buffered saline with 0.02% w/v calcium chloride, 0.02% w/v magnesium chloride and 0.1% w/v bovine serum albumin.

Lectin buffer 2 - as for lectin buffer 1, without the bovine serum albumin.

Gel electrophoresis tank buffer - 3.03g of Tris, 14.4g of glycine, 1.0g of sodium lauryl sulphate per litre of distilled water, pH8.30.

Electroblotter transfer buffer - 100ml of gel electrophoresis tank buffer and 100ml of methanol added to 300ml of distilled water.

Gel sample buffer - 4 x stock solution: 50mM Tris-HCl, pH7.0, containing 4% w/v sodium lauryl sulphate, 0.1% w/v bromophenol blue, 5% v/v B-mercaptoethanol, 20% v/v glycerol. Stored frozen in aliquots.

Blocking buffer - A 10 x stock solution was first prepared: 60.5g Tris, 87.0g sodium chloride, 3.72g sodium EDTA, 25.0g gelatin, 2.0g thiomersal dissolved by heating in water. After cooling, 5.0ml of NP40 detergent was added, the pH adjusted to 7.4 and the volume adjusted to 1 litre. For use, the stock solution was diluted 1:8.60 in distilled water, and 4% v/v normal goat serum added.

BSA-Tris buffer - 20mM Tris-HCl, pH8.2, containing 0.5M sodium chloride, 0.05% w/v Tween 20 and 0.1% w/v bovine serum albumin.

DNA lysis buffer - 25mM Tris-HCl, pH8.0 containing 50mM glucose, 0.15M sodium chloride, 10mM sodium EDTA and 0.1 per cent w/v lysozyme (Sigma). Prepared fresh.

DNA wash buffer - 0.1M sodium acetate containing 50mM morpholine propane sulphonic acid (MOPS, Sigma), pH8.0.

Ribonuclease - 40 x stock solution: Tris-EDTA buffer (see below) containing 0.2% w/v ribonuclease A (Sigma). Heat inactivated (80°C, 10 minutes) prior to use.

Chloroform - A mixture of chloroform and isoamylalcohol (24:1 v/v), stored at 20°C.

Phenol - liquid phenol (BDH Chemicals, U.K.) stored frozen, with 0.1% w/v hydroxyquinoline added when melted. The phenol was then extracted with an equal volume of 1.0M Tris-HCl, pH8.0 in a glass separating funnel, then with 0.1M Tris-HCl, pH8.0 containing 0.2% B-mercaptoethanol.

DNA sample buffer - 6 x stock solution: 30mM sodium acetate containing 15% w/v Ficoll 400 (Pharmacia Fine Chemicals, Sweden), 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol.

Tris-acetate-EDTA buffer - 50 x stock solution: 242g Tris was mixed with 100ml of a 0.5M sodium EDTA solution. 57.1ml of acetic acid was added to pH8.0 and the volume adjusted to 1 litre.

Tris-EDTA buffer - 10mM Tris-HCl, pH8.0, containing 1mM sodium EDTA.

Alkaline-SDS solution - 0.2N sodium hydroxide containing 1% w/v sodium lauryl sulphate. Prepared fresh.

Concentrated acetate solution - 3M of sodium acetate mixed with 100ml of water, the pH adjusted to 4.8 with acetic acid, and the volume adjusted to 1 litre.

Appendix 2 - Gels

Stock acrylamide solution - 154g acrylamide, 4g N,N methylene bisacrylamide, 10g mixed bed ion exchanger (Duolite, BDH Chemicals, U.K.), mixed slowly over heat until dissolved in 500ml of distilled water. Duolite removed by filtration, prior to use. T 30.8%, C 2.6%.

Stacking gel - 50ml Upper Tris (see below), 30ml stock acrylamide solution added to 120ml distilled water. Stored frozen in 20ml aliquots. T 4.5%.

Separating gels - T 7% - 50ml Lower Tris (see below), 46ml stock acrylamide solution added to 104ml distilled water.

- T 20% - 50ml Lower Tris, 132ml stock acrylamide solution, 30ml sucrose. Stored as above.

Upper Tris - 12.12g Tris, 0.8g sodium lauryl sulphate per 200ml distilled water, pH6.8.

Lower Tris - 36.4g Tris, 0.8g sodium lauryl sulphate per 200ml distilled water, pH8.8.

IEF stock acrylamide solution - 24.25g acrylamide, 0.75g N,N methylene bisacrylamide, 2.5g mixed bed ion exchanger (Duolite), mixed in 250ml of distilled water, and filtered, as above. T 10%, C 3%.

Appendix 3 - Stains

Modified acid-fast stain - Smear air-dried and heat-fixed onto a glass slide, allowed to cool. The slide is flooded with dilute carbol-fuchsin 5 minutes, rinse in water, 0.5% v/v acetic acid 18 seconds, rinse, methylene-blue 10 seconds, rinse, dry. Intracellular Campylobacter-like organisms - pink. Other bacteria, cells, blue.

Dilute carbol fuchsin is prepared by combining 5g of basic fuchsin powder, 25g crystalline phenol, 50ml absolute alcohol and 500ml of distilled water, then diluting it 10 times in water. Methylene blue is prepared by adding 300ml of a saturated solution of methylene blue in alcohol, to 1 litre of 0.01% w/v potassium hydroxide.

Morrissey silver stain for gels - The gel was immersed in a solution of 50% v/v methanol, 10% v/v acetic acid in water for 30 minutes, then 5% v/v methanol, 7% v/v acetic acid in water, for 30 minutes, then 10% v/v glutaraldehyde in water for 30 minutes. After extensive washing in distilled water, the gel was immersed in 0.1% w/v silver nitrate in water for 30 minutes, then rinsed once in water, once in developer, then immersed in developer made up of 3% w/v sodium carbonate in water with 0.018% v/v formalin, until bands appeared. The reaction was stopped by adding 2.3M citric acid (5ml per 100ml developer). If gels were to be dried, they were rinsed in water, fixed in a 1:9 dilution of "Ilfofixer" in water, then immersed in a solution of 20% v/v methanol, 5% v/v acetic acid, 1% w/v glycerine in water, prior to drying.

White cell stain - 5% v/v acetic acid, 2% v/v ethanol, 2% v/v methylene blue (see above) in distilled water. For use a 0.01ml sample was added to 0.09ml stain and examined.

Trypan blue stain - 1% w/v trypan blue powder in phosphate-buffered saline. For use a 0.01ml sample was added to 0.01ml stain and examined. Viable cells considered to be capable of excluding stain.

Appendix 4 - Cell culture

Glutamine - 100 x stock solution: 2.923g L-glutamine per 100ml phosphate-buffered saline. "Filter-sterilized" by passing solution through a 0.22µm sterile filter into a sterile bottle.

Penicillin/streptomycin - 100 x stock solution: 3.5g benzyl penicillin (potassium salt), 5.0g streptomycin sulphate per 500ml phosphate-buffered saline. Filter-sterilized.

Hypoxanthine-thymidine - 50 x stock solution: 0.340g hypoxanthine, 0.097g thymidine dissolved by heating in 500ml of RPMI 1640 medium (Gibco, U.K.). Filter-sterilized.

Aminopterin - stock solution: 0.016g aminopterin added to 50ml distilled water, heated to 45°C. A few drops of 0.1M sodium hydroxide was added to dissolve the aminopterin, then the solution made up to 100ml.

Hypoxanthine-aminopterin-thymidine - 50 x stock solution: hypoxanthine, thymidine dissolved as above, in 475ml of medium, cooled, then 25ml of aminopterin stock solution added. Filter-sterilized.

Mercaptoethanol - 100 x stock solution: 0.140ml of B-mercaptoethanol added to one litre of phosphate-buffered saline. Autoclaved.

Polyethylene glycol - prepared separately: polyethylene glycol, MW 4,000 (Sigma Chemical Co., U.K.) 50% w/v in RPMI 1640 medium; 2M Tris-HCl pH8.5. Filter-sterilized. Just prior to use, 0.01ml of Tris added to 1.0ml of polyethylene glycol.

Cell freezing mixture - Fresh cells suspended at a concentration of 10^6 cells ml⁻¹ in a solution of 50% v/v foetal calf serum, 10% v/v dimethyl sulphoxide in RPMI 1640 medium. Cells frozen at -70°C for 48 hours, then suspended in liquid nitrogen.

Agar medium for cloning - 40ml of 5% w/v Difco bacto agar (Difco, U.K.) in water, is dissolved by heating to 45°C, then added to 360ml of warm RPMI 1640 medium containing 20% v/v foetal calf serum, glutamine, penicillin/streptomycin (and HAT if in use). Kept at 45°C prior to pouring.

Amphotericin - 0.050g amphotericin (Fungizone, Squibb, U.S.A.) added to 10ml sterile, distilled water. This solution added to 250ml sterile phosphate-buffered saline gives a 100 x stock solution of amphotericin, Not filtered.

Appendix 5 - Methods

Preparation of Campylobacter-like organisms - Two to four grammes of mucosa was thawed (if frozen), washed twice by centrifugation (400g, 10 minutes) in 100ml of diluent (see below). The pellet was either i) resuspended in 10ml of diluent, then disaggregated by homogenization at 13,000 rpm for 30 seconds in an overhead blender (M.S.E. Instruments, U.K.), followed by grinding in a Jencon grinder; or ii) disaggregated by mixing in 10ml of 0.75% w/v trypsin in diluent, for 40 minutes at 37°C.

The suspension was diluted to 20ml in diluent, then homogenized as described above, to release intracellular organisms. After centrifugation (4,000g, 20 minutes), the pellet was resuspended in 120ml of diluent (with formalin if required). After grinding in the Jencon grinder, the suspension was filtered sequentially through a coarse prefilter (Whatman GFD, 2.5µm pore size) fine prefilter (Whatman GFB, 1.0µm pore size) then 1.2µm pore size filter (Millipore, U.K.) and 0.8µm filter (Millipore, U.K.), all 47mm diameter.

For use in antigenic analysis, the final filtrate was centrifuged (4,000g, 20 minutes) and the pellet resuspended in diluent to a suspension approximating Brown's opacity tube no. 2, in a Nephelometer.

Diluent was usually PBS (see Appendix 1), with 0.3% formalin as preservative if required.

For material to be subsequently passed through a lectin column (Chapter 5) diluent was lectin buffer 2 (see Appendix 1).

Affinity purification of IgG antibodies - One millilitre of protein A-Sepharose 4B (Sigma) in a vertical column was washed in 20ml of buffer A: 0.05M Tris-HCl, pH8.2 with 0.15M sodium chloride. Five millilitres of buffer B: 1M Tris-HCl, pH8.2, was added to 100ml of hybridoma supernatant fluid and the mixture passed slowly through the column.

After washing the column with 10ml of buffer A, the IgG was eluted with 4ml of 0.05M sodium citrate - citric acid pH3.5, with 0.15M sodium chloride, into 2ml of buffer B. The eluate was dialyzed against buffer B (16 h). The column was regenerated by washing with 10ml of buffer B (Kohler, 1981).

An alternative to buffers A and B is 1.5M glycine-OH, pH8.9, with 3M sodium chloride.

Ammonium sulphate purification of IgG antibodies - powdered ammonium sulphate was added to serum, at 0.29g ml⁻¹, to give 50% saturation. After mixing for 16 hours at 4°C, the suspension was centrifuged (12,000g, 20 minutes) and the pellet resuspended in distilled water. After dialysis (16 hours, 4°C) against distilled water, the fluid was freeze-dried as described in Chapter 5. The resultant immunoglobulin powder was used fresh.

Protein determination - 0.1ml of sample, or standard (bovine serum albumin in 5 dilutions) was mixed with 2ml of BCA working reagent (see below), and tubes incubated at 37°C for 30 minutes. Absorbance of tubes and blank tube measured at 562nm at 20°C. Protein concentrations in sample determined from a standard curve.

Working reagent = 50 parts sodium carbonate, sodium bicarbonate, sodium tartrate and BCA reagent - bicinchoninic acid, in 0.1N sodium hydroxide, to one part copper sulphate. (Pierce Chemical Co., U.S.A.).

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S. McOrist, R. Boid, G. H. K. Lawson, I. McConnell

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PROLIFERATIVE enteropathy or enteritis is a pathologically defined disease affecting a variety of mammals, frequently being clinically important in pigs and hamsters (Jacoby and Johnson 1981, Rowland and Lawson 1986). Consistent features are an initial phase of hyperplasia of the crypt cells of the intestinal epithelium (particularly those in the ileum) and the occurrence of intracytoplasmic, non-membrane bound campylobacter-like organisms within enterocytes in affected portions of intestine (Rowland and Lawson 1974, Johnson and Jacoby 1978). Other pathologically similar proliferative enteropathies include necrotic enteritis and a haemorrhagic enteropathy (Rowland and Lawson 1975). The identity of these campylobacter-like organisms has not been resolved by animal experiments, nor by immunofluorescence assays incorporating polyclonal rabbit anti-serum (McCartney and others 1984, Boosinger and others 1985, Lawson and others 1985), although *Campylobacter mucosalis*, *Chyointestinalis* and *C jejuni* have been regularly cultured from the lesions (Lawson and Rowland 1974, Lentsch and others 1982, Gebhart and others 1983). Pig and hamster organisms appeared to express a similar antigen (Lawson and others 1985) but there is no certainty that all the conditions are associated with a common organism. The production of monoclonal antibodies specific to intracellular campylobacter-like organisms is reported here.

The intestinal mucosa was collected from a pig, designated 284/86, affected by histologically confirmed proliferative haemorrhagic enteropathy and stored at -70°C . The mucosa was thawed, washed twice in 0.1 M phosphate buffered saline (PBS), pH 7.4 (PBS) and incubated in 0.75 per cent trypsin in PBS at 37°C for 40 minutes to disperse the cells. Intracellular organisms were released from cells by homogenisation for 30 seconds in a blender. The resulting homogenate was diluted in PBS and filtered sequentially through 2.5 μm , 1.2 μm , 0.8 μm and 0.65 μm pore diameter filters (Millipore). Smears of this filtrate revealed numerous campylobacter-like organisms, by modified acid-fast staining, however, only 10^2 *C coli*/ml were cultured. The filtrate was applied to a column of wheatgerm (*Triticum vulgaris*) agglutinin, insolubilised on cross-linked 4 per cent beaded agarose (Sigma) in PBS, which had been previously shown to react with *C coli* strains isolated from the homogenate. BALB/c mice were immunised with intact campylobacter-like organisms (first intramuscularly in Freund's complete adjuvant, then alone intravenously) either from the unbound fraction or from the fraction collected following elution of the column with N-acetyl D-glucosamine. Each mouse serum was tested by indirect immunofluorescence assay, incorporating as the test antigen a 0.65 μm filtrate from a separate intestinal mucosa, designated 1269/76, affected by proliferative enteritis. Mice given affinity purified bacteria from either fraction developed serum titres to the test antigen of 1:100 to 1:200. Each spleen was removed four days after the last booster injection and fused with NSO mouse myeloma cells using standard techniques (Galfre and Milstein 1981). Due to technical problems, only the fusion derived from the mouse immunised with the eluted fraction was further processed. Hybridomas showing positive reactions in the immunofluorescence assay described above were cloned by limiting dilution and in soft agar, selected clones were then grown in bulk in vitro.

Reactive supernatant fluid was tested in further immunofluorescence assays, incorporating as the test antigen filtered suspensions in PBS-formalin of mucosae 284/86, 1269/76 or mucosa from a healthy, *Campylobacter* species culture negative pig (204/79). Also, suspensions of the following cultures of campylobacter strains isolated from proliferative enteropathies were tested: *C mucosalis* (n=2); *Chyoin-*

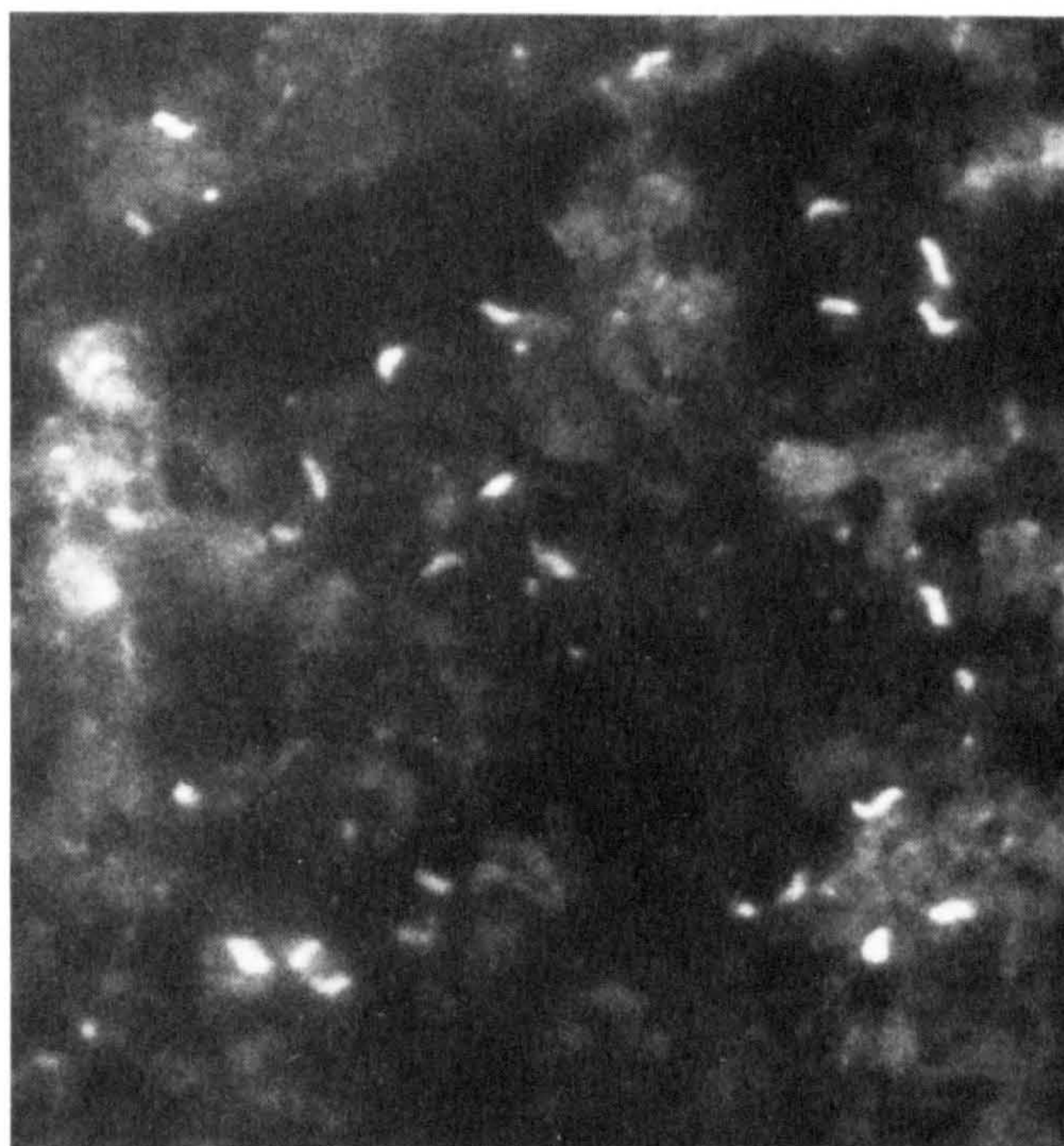


FIG 1: Photomicrograph of a faecal smear from a pig affected by proliferative haemorrhagic enteropathy, stained with monoclonal antibody 4F5 in an indirect immunofluorescence assay. Numerous fluorescing curved bacilli evident. $\times 600$

testinalis (n=2); *C fetus* (n=1); *C jejuni* (n=2) and *C coli* (n=3). Faecal smears from pigs (n=6) and hamsters (n=4) affected by proliferative enteritis and from healthy pigs (n=5) and hamsters (n=2) were also tested. Histological sections of the intestines from healthy pigs (n=4) and hamsters (n=3), also pigs (from a number of separate farms) affected by proliferative intestinal adenomatosis (n=3) and necrotic enteritis (n=3), proliferative haemorrhagic enteropathy (n=4) and hamsters affected by proliferative enteritis (n=7), were trypsin digested as described by Lawson and others (1985). Supernatant fluid was the first antibody in an indirect immunofluorescence assay of mucosal filtrates, cultures, sections and faecal smears. Identical suspensions of filtered mucosae and *Campylobacter* species to those outlined above, were used as the solid phase in an enzyme-linked

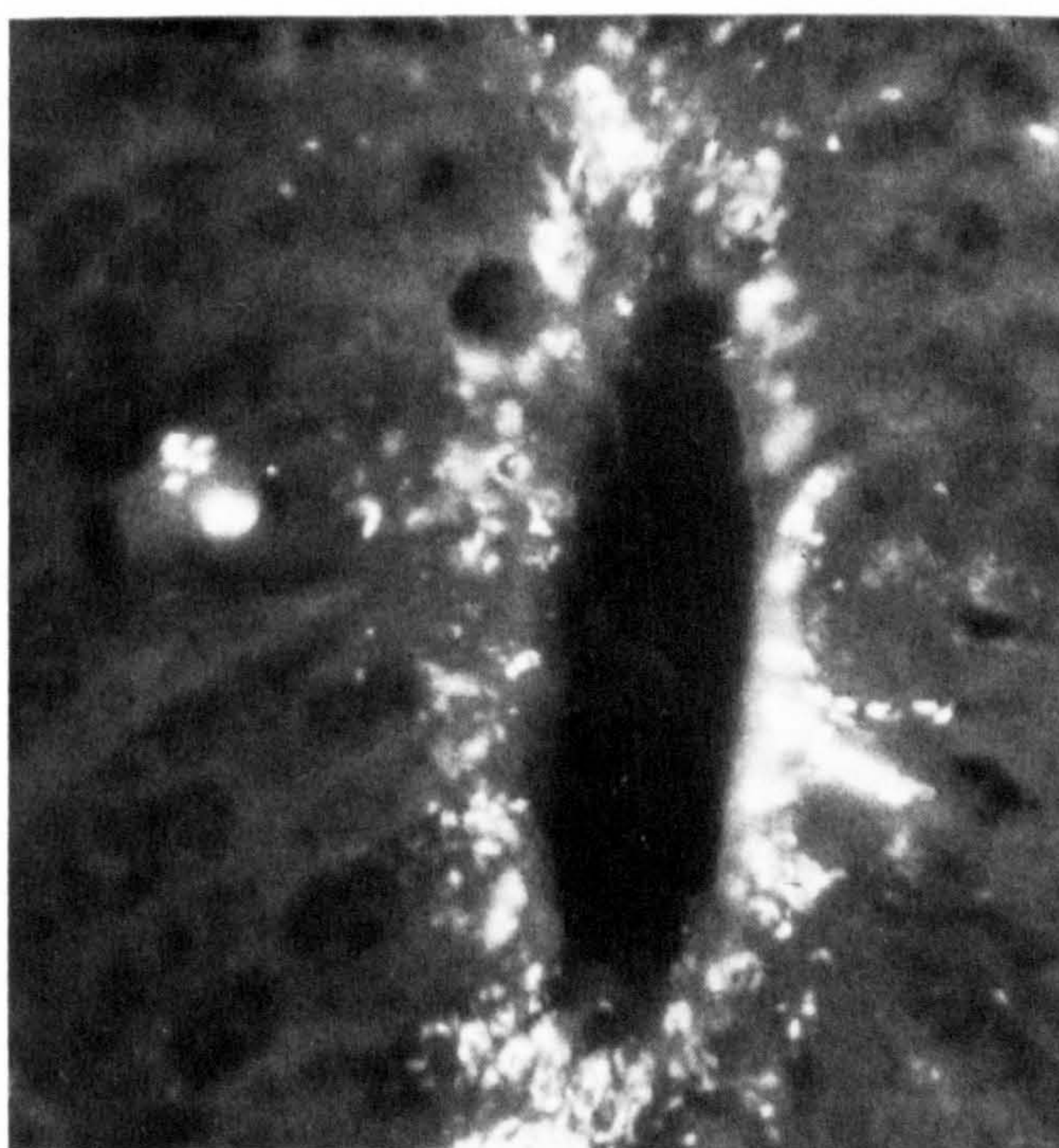


FIG 2: Photomicrograph of the ileum of a hamster affected by proliferative enteritis stained with monoclonal antibody IG4 in an indirect immunofluorescence assay. Fluorescing curved bacilli within the apical cytoplasm of enterocytes. $\times 600$

S. McOrist, G. H. K. Lawson, I. McConnell, Department of Veterinary Pathology, University of Edinburgh, Edinburgh
R. Boid, Centre for Tropical Veterinary Medicine, Easter Bush, Roslin, Midlothian

immunosorbent assay (Voller and others 1980) incorporating the supernatant fluids as the first antibody.

Seventy-two hybridomas were produced on which two (IG4, 4F5) were positive on initial screening against the test antigen. These were cloned, isotyped as IgG3 and both clones had similar reactions thereafter. Suspensions of all affected mucosae, and six of the 10 faecal samples from affected animals, showed brightly fluorescing curved bacilli, at supernatant fluid dilutions (in PBS) up to 1:100 (Fig 1). Intestinal sections from affected pigs and hamsters had numerous fluorescing curved bacilli within the apical cytoplasm of proliferative enterocytes (Fig 2). Fluorescent particles were also evident in mucosal macrophages. No such reactions were observed (at any dilutions) in faeces or sections from healthy animals, nor with any *Campylobacter* species culture tested.

Similarly, enzyme immunoassays of mucosa from affected pigs, at supernatant fluid dilutions of up to 1:1000 gave positive colour reactions (optical density 0.2 to 0.35). Negative reactions (optical density ≤ 0.1) were observed with all other antigen preparations, at supernatant fluid dilutions greater than 1:2.

Campylobacter-like organisms within enterocytes in proliferative enteritis have an outer membrane component that is apparently specific for these organisms, and the organism within lesions in pigs and hamsters contain an identical antigen. Thus proliferative enteritis is associated with a campylobacter-like organism apparently markedly different to known *Campylobacter* species. The various forms of proliferative enteropathy in pigs, namely, necrotic enteritis, proliferative haemorrhagic enteropathy and intestinal adenomatosis, all also contain intracellular campylobacter-like organisms carrying a common antigen. Thus if campylobacter-like bacteria are the cause of proliferative intestinal lesions, a common or closely related organism is involved and these bacteria are distinct from those *Campylobacter* species currently recognised.

A further significant result is the demonstration of organisms specific for proliferative enteritis in the faeces of live animals, later confirmed as having proliferative enteritis at autopsy. This offers a great advance in clinical diagnosis of the disease as serology or morphological examination of faeces have proved inadequate (Rowland and Lawson 1986). Furthermore, studies may now be possible which could help resolve the natural history of the disease.

Monoclonal antibodies to other intracellular bacteria such as *Mycobacterium leprae* have proven useful in immunodiagnosis and bacterial characterisation (Engers and others 1985).

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Ultrasonic appearance of the equine corpus haemorrhagicum

W. Edward Allen, P. E. Arbeid, K. Kooros, J. F. Pycock

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AFTER ovulation in the mare the collapsed ovarian follicle becomes redistended with blood to form a corpus haemorrhagicum (Harrison 1946). Luteal cells from the periphery of the corpus haemorrhagicum gradually replace the haematoma as the corpus haemorrhagicum matures and develops into a corpus luteum.

Using realtime, B mode, ultrasonography it is possible to monitor the development of ovarian follicles because follicular fluid is non-echogenic and appears black on the screens of conventional scanners (Ginther and Pierson 1984).

Circulating blood also appears black on ultrasonography (Edwards and Allen 1987) although various degrees of white (solid) scintillation are often seen when scanning the abdominal aorta (W. E. Allen and G. B. Edwards, unpublished observation). However, following ovulation in the mare the space previously occupied by the follicle usually exhibits a dense (white) appearance which has been attributed to rapid proliferation of luteal tissue (Pierson and Ginther 1985). The speed with which this apparent cellular development occurred did not correspond with the histological description of luteal growth, so a study of the ultrasonic characteristics of static equine blood was carried out. Equine jugular vein blood was collected into heparinised and non-heparinised containers and each sample was evacuated into the finger of a plastic 'rectal examination' sleeve. Both samples were then sequentially imaged by ultrasound in a bucket of water.

Both samples were initially echogenic, ie, completely different to circulating blood (Fig 1). With time it became apparent that plasma, serum and the non-cellular fibrinous thrombus that forms in clotted horse blood are all non-echogenic; sedimented and clotted cellular portions become intensely white (echogenic). The initial echogenicity

W. Edward Allen, P. E. Arbeid, K. Kooros, J. F. Pycock, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire

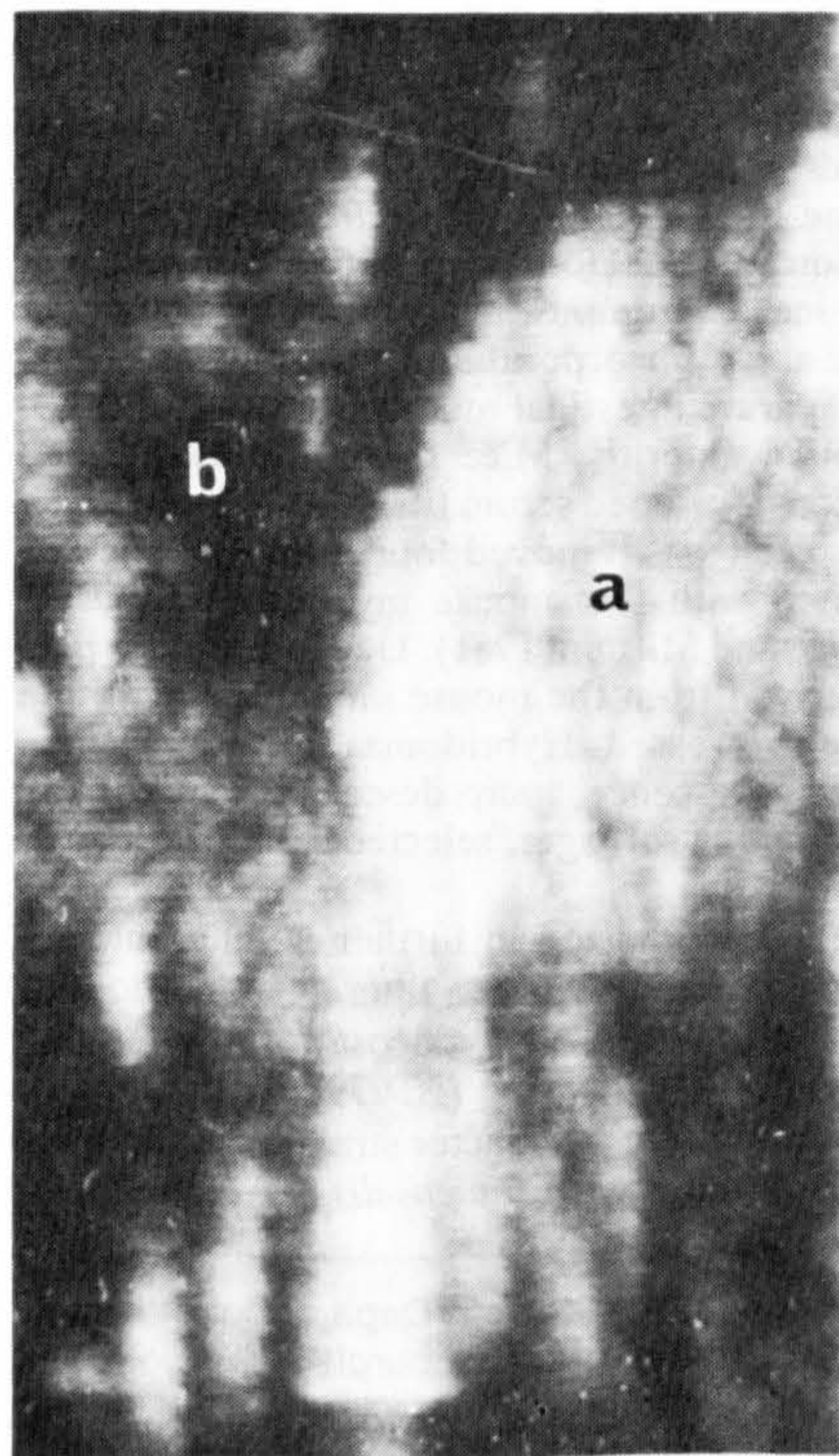


FIG 1: Ultrasonic appearance of fresh heparinised equine blood in a water bath (3.5 MHz). (a) echogenic blood in a plastic container, (b) water containing echodense particles and air bubbles

Possible Relationship of Proliferative Enteritis in Pigs and Hamsters

STEVEN McORIST AND GORDON H.K. LAWSON

Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Edinburgh EH9 1QH (Gt Britain)

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ABSTRACT

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Three- to six-week-old hamsters were orally inoculated with broths containing one of the following cultures: *Campylobacter mucosalis*; *C. hyointestinalis*; *C. coli*; *C. jejuni*, all of porcine proliferative enteritis origin, or else *C. jejuni* of hamster origin. Hamsters given the last of those organisms were shown to have colonisation of their intestines by *C. jejuni* and 36 of 40 developed an acute enteritis. Mild hyperplasia of enterocytes in ileal crypts was evident in one hamster 2 days after it was given *C. coli*. No other lesions were detected.

Further 3-week-old hamsters were orally inoculated with homogenised intestinal mucosa collected from 4 pigs (A–D) affected by proliferative enteritis. Lesions of proliferative enteritis were detected in 7 of 41 hamsters necropsied 10–21 days after being dosed with mucosae B or D. Marked hyperplasia of ileal enterocytes, associated with numerous intracellular *Campylobacter*-like organisms, were invariably detected in experimentally affected hamsters. No particular *Campylobacter* sp. was consistently isolated. None of the controls had demonstrable lesions.

The results suggested that cross-species transmission of proliferative enteritis was possible from pigs to hamsters. Therefore a common initiating or aetiological agent may be present. No specific organism was identified as filling this role by inoculation of hamsters with pure cultures.

INTRODUCTION

Proliferative enteritis presents as a similar clinical and pathological syndrome in hamsters (*Mesocricetus auratus*) and in pigs (Jacoby and Johnson, 1981). Affected weanling hamsters (3–10 weeks old) show hyperplasia of immature enterocytes in the ileum, with associated intracellular *Campylobacter*-like organisms. Naturally occurring disease in hamsters has only been reported from North America. Numerous experimental transmission studies have successfully reproduced the disease, by oral inoculation of weanling hamsters with

crude or filtered (0.65μ) ileal mucosa from affected hamsters (Frisk and Wagner, 1977; Jacoby, 1978). While *Campylobacter jejuni* has frequently been isolated from the intestines of affected hamsters, the disease has not been reproduced by oral inoculation of hamsters with this agent (Lentsch et al., 1982; Regina and Lonigro, 1982; Fox et al., 1986). *C. jejuni* occurs in the intestines of many healthy hamsters (Fox et al., 1981).

Only a small number of experimental transmission studies have successfully reproduced proliferative enteritis by oral inoculation of pigs with crude ileal mucosa from affected pigs (Roberts et al., 1977; Lomax et al., 1982). Both *C. mucosalis* and *C. hyointestinalis* have been consistently associated with the presence of lesions in pigs (Lawson and Rowland, 1974; Gebhart et al., 1983; Chang et al., 1984a). However these *Campylobacter* spp. (alone or in combination) have not reproduced proliferative enteritis in orally dosed pigs (McCartney et al., 1984; Chang et al., 1984b; Boosinger et al., 1985).

Therefore the identity of the intracellular *Campylobacter*-like organisms in affected pigs and hamsters is not known. Some immunofluorescence studies have suggested that the intracellular organism is antigenically different from known *Campylobacter* spp., and that the organism in pigs and hamsters is antigenically related (Jacoby and Johnson, 1981; Lawson et al., 1985). This study examined possible relationships between *Campylobacter* organisms and proliferative enteritis.

MATERIALS AND METHODS

Hamsters

Hamsters were obtained from 2 sources, a small closed colony *C. jejuni* free, the progeny of which were used for experimental studies, and the parent colony which was infected with this organism. Complete diets of either Mouse-Hamster Diet (Special Diet Services Ltd., Witham, Essex, U.K.) or Mouse Chow (Ralston Purina, St. Louis, Missouri, U.S.A.), containing 5% and 3% fibre, respectively, were given ad libitum to all hamsters. Faeces and intestinal tract were regularly monitored for the presence of *C. jejuni* by culture on selective media (Skirrow, 1977). All hamsters were weaned and dosed at 3 weeks of age, except for Groups 1 and 2, which were dosed at 6 and 5 weeks, respectively.

Campylobacter spp. inocula

Each inoculum of 0.5 ml of broth culture was administered orally via a blunt-ended 16-gauge needle. Controls were not dosed.

The identity of each isolate was confirmed by biochemical and slide agglutination reactions typical of each *Campylobacter* sp. (Hebert et al., 1982; Lawson et al., 1976, 1985; Gebhart et al., 1985; Roop et al., 1985). Each isolate had

TABLE I

Characterization of porcine proliferative enteritis tissue used as inocula

Pig tissue inocula	Age of pig	Campylobacter spp. detected	Concentration ^a	Smear ^b	Pathology
A ^c	6 weeks	<i>C. mucosalis</i>	4.6	+++	Proliferative enteritis
B ^c	8 weeks	<i>C. hyointestinalis</i>	2.0	+++	Proliferative enteritis
		<i>C. mucosalis</i>	5.8		
		<i>C. hyointestinalis</i>	2.6		
C	30 weeks	<i>C. coli</i>	2.0	+	Proliferative ^d haemorrhagic enteropathy
		<i>C. fetus</i>	2.5		
		<i>C. coli</i>	5.5		
D	40 weeks	<i>C. coli</i>	3.5	+++	Proliferative haemorrhagic enteropathy
		<i>C. fetus</i>	2.0		

^aBacterial concentrations expressed in log₁₀ ml⁻¹ of crude inoculum.
^bNumber of *Campylobacter* spp. detected in a smear of fresh mucosa stained by modified acid fast. +++ = > 100; ++ = < 100 > 10; + = < 10 > 0 per high-power field.
^cCrude inocula A and B diluted 1:10 in phosphate-buffered saline prior to administration.
^dFocal lesions only.

been subcultured 5–10 times, and stored in glycerinated serum at –70°C. For each experiment a culture was transferred to Brucella broth (Difco, U.K.), incubated at 37°C in an atmosphere of 3% or 6% oxygen, 10% carbon dioxide, 70% hydrogen with nitrogen for 48 h, and given to hamsters as described above. Viable counts of *Campylobacter* sp. in each inoculum, as calculated by the method of Miles and Misra (1938), were log₁₀ 8.3 ml⁻¹ for *C. mucosalis* 253/72, log₁₀ 9.6 ml⁻¹ for *C. hyointestinalis* 124/73, log₁₀ 7.0 ml⁻¹ for *C. jejuni* 1268/84, log₁₀ 7.0 ml⁻¹ for *C. coli* 1268/84 K. These 4 isolates were originally from 3 porcine ilea affected with histologically confirmed proliferative enteritis (Rowland and Lawson, 1986). *C. jejuni* 664/83 was originally isolated from the ileum of a healthy hamster. It was given at a dose of log₁₀ 8.9 ml⁻¹.

Proliferative enteritis inocula

Intestinal mucosa affected by proliferative enteritis was collected aseptically from 4 pigs (A–D), see Table I. The method of diagnosis was described above. Samples of mucosa were stored at –70°C prior to use. Upon thawing, mucosal samples were weighed, washed in phosphate-buffered saline, homogenised to a fluid consistency and 1–1.5 gm were administered orally to each hamster as described above. A 1-ml sample of each inoculum was diluted for viable counts

and incubated on Skirrow's medium (Skirrow, 1977) and RNBGT medium (McCartney et al., 1984) at 37°C in an atmosphere of 3% or 6% oxygen, 10% carbon dioxide and 70% hydrogen with nitrogen. *Campylobacter* sp. isolations were identified as described above. For tissues B, C and D, separate groups of hamsters were concurrently given 0.5 ml of a broth containing \log_{10} 7.5 to 8.5 ml^{-1} of *C. jejuni* 664/83, prepared as described above. Controls were not dosed.

Necropsy procedures

Pathology

The small intestine from jejunum to lower ileum, and portions of caecum and colon, were collected into buffered formalin at necropsy. Subsequently the fixed small intestine was opened and rolled onto a wooden stick, prior to embedding, enabling the entire intestine collected to be examined on one slide.

Four μm sections were routinely prepared and stained, with a separate silver impregnation stain (Young, 1969). The ratio of crypt to villus height was estimated using a measured objective eyepiece. Ten crypt/villus units were measured per section, and a mean value calculated.

Bacteriology

For experimental groups 1–4 and 6–8, see Table II, the terminal 5 cm of ileum from each hamster was opened, washed in saline and a sterile swab rubbed into the mucosa. Swabs were spread onto selective media and incubated as described above.

For experimental groups 5 and 9–14, a 1-cm portion of ileum from each hamster was removed aseptically, homogenised and diluted in known volumes of phosphate buffered saline. The identity and concentration of *Campylobacter* spp. in these dilutions was determined as described above.

Indirect immunofluorescence

Formaline-fixed unstained sections of intestine from each hamster were trypsin-digested, and prepared for immunofluorescence as described by Lawson et al. (1985). Primary rabbit antisera used were those prepared by the method of Lawson et al. (1976) in our laboratory, against *C. jejuni* 664/83, and intracellular *Campylobacter*-like organisms purified from lesions of proliferative enteritis in a pig 1269/76 (Lawson et al., 1985).

RESULTS

No *Campylobacter* sp. was detected in the faeces of any hamster prior to experimentation or in control hamsters at necropsy. All hamsters remained clinically healthy throughout.

One hamster in each of experimental groups 7 and 12 (see Table II) had

TABLE II

Features of hamsters dosed with *Campylobacter* spp. and/or porcine proliferative enteritis tissue

Experi- mental group	Inoculum	No. of hamsters dosed	Date(s) of ^a necropsy	Histological changes in intestines	Mean crypt to villus ratio	Intestinal culture
1	<i>C. mucosalis</i>	4	23	NAD 4/4	ND	NSI
2	<i>C. hyointestinalis</i>	3	9	NAD 3/3	ND	NSI
3	<i>C. jejuni</i> (pig)	4	2,9	NAD 4/4	0.26	NSI
4	<i>C. coli</i>	4	2	Crypt hyperplasia 1/4	0.45	NSI
			2,9	NAD 3/4	0.35	NSI
5	<i>C. jejuni</i> (hamster)	14	5,8,12,18	Acute enteritis 14/14	0.35	<i>C. jejuni</i>
6	Pig Tissue A	4	23	NAD 4/4	0.60	NSI
7	Pig Tissue B	20	23	Proliferative enteritis 1/20	0.49	NSI
			14,23	NAD 19/20	0.26	NSI
8	B + <i>C. jejuni</i> (hamster)	8	14,23	Acute enteritis 8/8	0.34	<i>C. jejuni</i>
9	Pig Tissue C (hamster)	5	23	NAD 5/5	0.32	NSI
10	C + <i>C. jejuni</i> (hamster)	10	7,23	Acute enteritis 10/10	0.35	<i>C. jejuni</i>
11	Controls for 1-10	37	2,8,14,23	NAD 37/37	0.28	NSI
12 ^b	Pig Tissue D	5	10,21	Proliferative enteritis 2/5	0.65	NSI
			10,21	NAD 3/5	0.45	NSI
13 ^b	D + <i>C. jejuni</i> (hamster)	8	10,21	Proliferative enteritis 4/8	0.85	<i>C. jejuni</i>
			21	Acute enteritis 4/8	0.35	<i>C. jejuni</i>
14 ^b	Controls for 12-13	6	10,21	NAD 6/6	0.38	NSI

^aDates of necropsy expressed in no. of days after dosing. At least 2 hamsters from each group were necropsied on each date.

^bThese groups were fed Mouse Chow (Purina) all others fed Mouse-Hamster Diet.

NSI = no significant bacteria isolated; ND = not done; NAD = no abnormality detected.

gross thickening of the mucosa of their colon and ileum, respectively, at 21 days post-infection. No other gross lesions were detected.

Campylobacter spp. inocula

All hamsters dosed with *C. jejuni* 664/83 had varying degrees of localised acute intestinal inflammation. Mucosal infiltration by neutrophils, lymphocytes and macrophages, and crypt lumina containing numerous *Campylobacter*-like organisms, were consistent features; the latter being readily visible on silver stains. Also, fluorescing curved bacilli were evident in these crypt lumina in sections stained with antiserum raised against *C. jejuni*, see Fig. 1. Sections stained with antiserum against *Campylobacter*-like organisms 1269/76 showed no fluorescence. *C. jejuni* was recovered from all hamsters dosed with this organism alone, in concentrations of log₁₀ 7.5 per cm ileum where measured.

One hamster dosed with *C. coli* had moderate crypt cell hyperplasia in its jejunum and ileum at necropsy 2 days post-infection. However, its crypt/villus

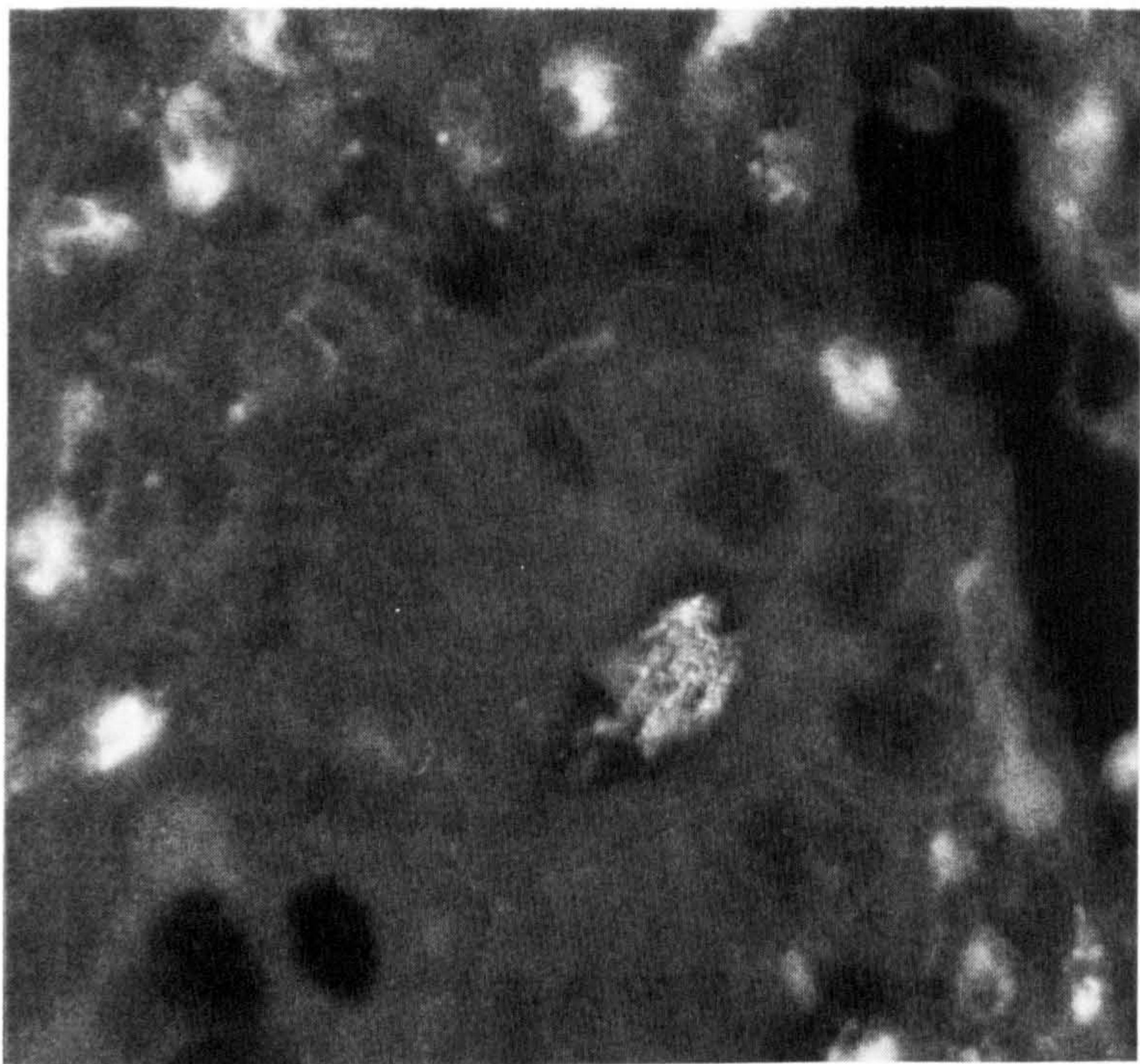


Fig. 1. Indirect immunofluorescent stain of the ileum of a group 5 hamster, 8 days after oral inoculation with *C. jejuni* (hamster origin). Numerous fluorescent curved bacilli are present in crypt lumina. Rabbit antiserum 664/83 *C. jejuni*, 1:40 dilution.

ratio was not markedly greater than that of other bacteria-dosed hamsters, see Table II. All hamsters dosed with *C. coli* had crypt lumina containing numerous curved bacilli, and fluorescing curved bacilli were evident in these crypt lumina in sections stained with *C. jejuni* antiserum.

No intracellular organisms were detected, by silver stain or immunofluorescence assays with either antiserum, in any hamster in these groups. No other fluorescence, isolations or lesions were evident, see Table II.

Proliferative enteritis inocula

Lesions of proliferative enteritis were detected only in hamsters dosed with tissue. Lesions in the 7 affected hamsters in groups 7 and 12-13 were confined to the colon and ileum, respectively. Marked hyperplasia of crypt enterocytes, with numerous mitotic figures were evident. Numerous intra-cytoplasmic *Campylobacter*-like organisms were detected within enterocytes in affected portions of intestine, both by silver stains and by immunofluorescent staining with antiserum 1269/76, see Fig. 2.

These hamsters with developed lesions had few curved bacilli within local

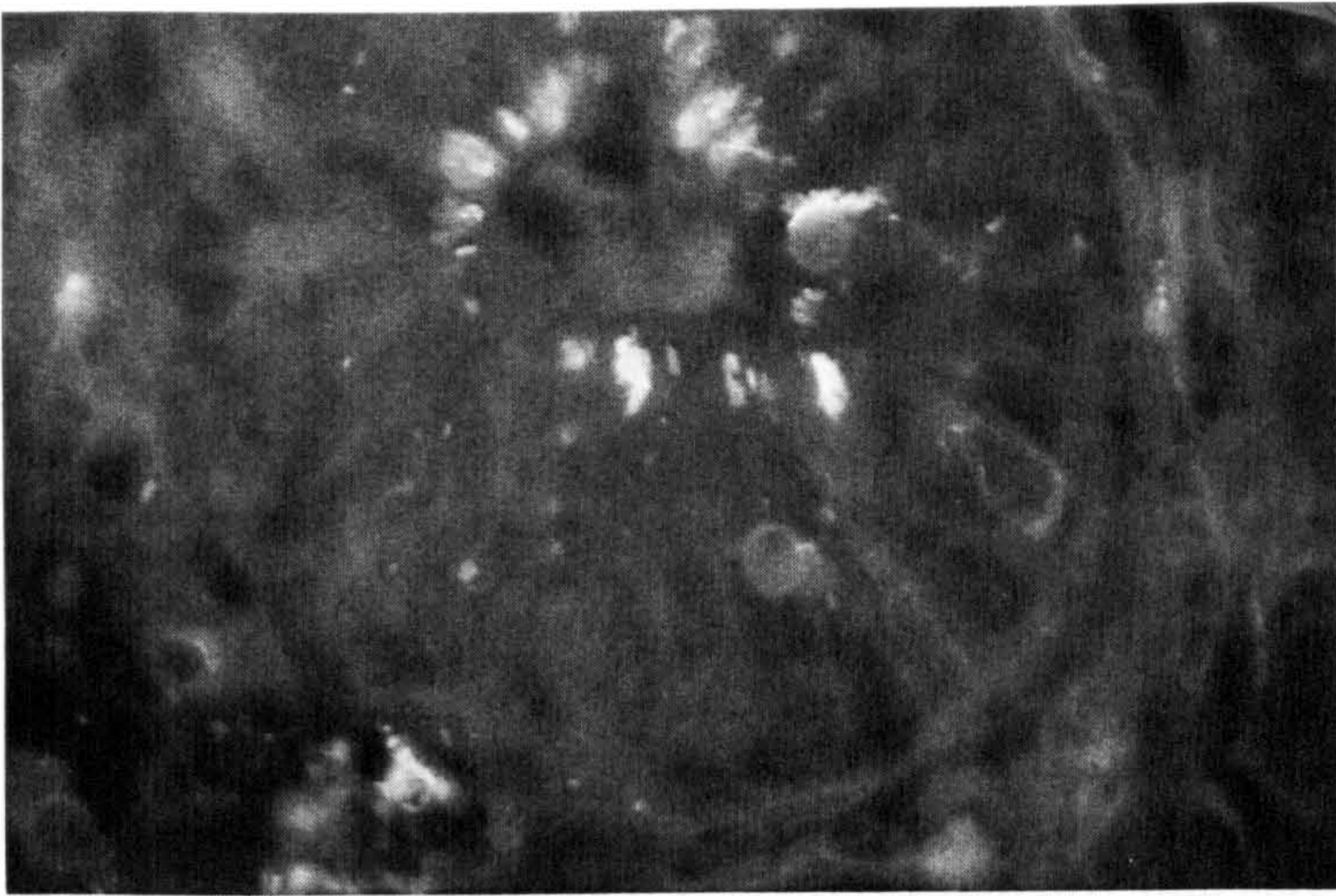


Fig. 2. Indirect immunofluorescent stain of the ileum of a group 13 hamster 21 days after oral inoculation of porcine proliferative enteritis material. Numerous fluorescent *Campylobacter*-like organisms in the apical cytoplasm of crypt enterocytes. Rabbit antiserum 1269/76 *Campylobacter*-like organisms, 1:150 dilution.

intestinal crypt lumina, and no fluorescence was evident in sections stained with *C. jejuni* antiserum.

However, *C. jejuni* was recovered from all hamsters dosed with both *C. jejuni* and porcine tissue (except for 4 in group 8), in concentrations of \log_{10} 6.0–7.0 per cm ileum where measured. All such hamsters also developed mucosal changes, similar to those described for hamsters dosed with *C. jejuni* alone. Fluorescing curved bacilli were also evident in crypt lumina of all hamsters (other than those with proliferative enteritis) dosed with *C. jejuni* and porcine tissue, in sections stained with *C. jejuni* antiserum. Spiral and curved bacilli were also detected in intestinal crypt lumina in silver-stained sections in 15 control hamsters; these organisms did not react with *C. jejuni* antisera in immunofluorescence assays. No other fluorescence, isolations or lesions were evident, see Table II.

DISCUSSION

Weanling hamsters proved to be susceptible to the agent of porcine proliferative enteritis by cross-species transmission. Several factors appeared to enhance this susceptibility; animals on the Purina diet and receiving a concentrated inoculum of Pig D tissue showed the highest incidence of the disease. The disease which appeared in dosed hamsters was nearly identical to the naturally occurring one in pigs (Rowland and Lawson, 1986) and hamsters (Jacoby and Johnson, 1981). Oral dosing of certain *Campylobacter* spp. cul-

tured from pigs or hamsters did not appear to be capable of reproducing proliferative enteritis.

The variation in the infectivity of the various porcine tissue inocula may partly be due to a dietary effect. Although the Purina diet was only used with Pig D tissue, it appeared to enhance the infectivity, a phenomenon similar to previous findings in groups, with both hamster proliferative enteritis and murine colonic hyperplasia transmission experiments, on various diets (Barthold et al., 1977; Jacoby and Johnson, 1981). The "successful" diets may damage crypt cells or enhance their ability to take up antigens, provide substrates for growth of *Campylobacter* or alter local enzymes or redox potentials. Also pig Tissues A and B were diluted prior to use and Tissue C had fewer *Campylobacter*-like organisms evident, suggesting that for reproduction of proliferative enteritis, a dose containing numerous intracellular *Campylobacter*-like organisms is required. Similar conclusions were made from hamster tissue transmission studies (Frisk and Wagner, 1977; Jacoby, 1978).

The identity of the intracellular organisms remains unresolved. No hamster has developed proliferative enteritis following inoculation of pure cultures of *Campylobacter* spp. in this, or in previous studies (Lentsch et al., 1982; Regina and Lonigro, 1982; Fox et al., 1986). The failure to isolate *C. mucosalis* or *C. hyointestinalis* from any hamster suggests that these organisms are not associated with proliferative enteritis in this species. The inoculations of *C. jejuni* of hamster origin, or *C. coli* of pig origin, consistently produced some crypt cell hyperplasia and acute enteritis, but this may have been a non-specific reaction, as no intracellular bacteria were observed. Furthermore, the failure of hamsters in Groups 8 and 10 to develop proliferative enteritis suggests that no relationship exists between *C. jejuni* and an unidentified factor in proliferative mucosa, e.g. a virus, in the aetiology of proliferative enteritis.

The exact relationship of the spiral bacteria within crypt lumina and those within adjacent cells in experimentally reproduced proliferative enteritis was not clear. Immunofluorescent staining suggested that the intracellular bacteria were antigenically different from *C. jejuni*; however *C. jejuni* was recovered in significant numbers from some affected ilea, and immunofluorescent staining identified *C. jejuni* in crypt lumina in group littermates of some affected hamsters, but not in affected hamsters. However, other populations of *Campylobacter* organisms were also possibly involved, including *C. coli* or the intracellular *Campylobacter*-like organisms in the original inocula. It is not possible to assign particular roles to any of these populations at this stage.

While it is likely that some *C. jejuni/coli* strains can colonise the mucus of intestinal crypts, and are difficult to distinguish morphologically from spiral bacteria present in the crypts of healthy animals (Lee et al., 1986) our cultural and immunofluorescent results suggest that *C. jejuni* can form significant colonies in ileal crypts. Acute inflammation has been reported in previous exper-

imental studies of *C. jejuni* infection of the hamster (Regina and Lonigro, 1982).

It is possible that only a particular strain of one or more *Campylobacter* sp. is capable of crypt cell entry and subsequent proliferation, and that this property is readily lost in vitro. *Citrobacter freundii* 1140 was the only strain of 23 tested of this organism capable of causing murine colonic hyperplasia (Barthold et al., 1977). The crypt cells of weanling hamsters and pigs appear to be naturally susceptible to a common agent, and entry of the crypt cells by *Campylobacter* sp. is an essential stage that may not always take place. An alternative explanation is that the causative agent is an obligate intracellular parasite, whose presence is obscured by the numerous *Campylobacters* that can be recovered from the alimentary tract.

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